P.D. Biología Celular y Molecular
Facultad de Ciencias
Universidad de Málaga

TESIS DOCTORAL

Analysis of the microbial community of a suppressive soil from an avocado crop

Carmen Mª Vida Hinojosa

Directores
Dr. Francisco M. Cazorla López
Dr. Antonio de Vicente Moreno

MARZO 2017

AUTOR: Carmen María Vida Hinojosa

http://orcid.org/0000-0002-6907-7255

EDITA: Publicaciones y Divulgación Científica. Universidad de Málaga

Departamento de Microbiología

Facultad de Ciencias

TESIS DOCTORAL

ANALYSIS OF THE MICROBIAL COMMUNITY OF A SUPPRESSIVE SOIL FROM AN AVOCADO CROP

Carmen Mª Vida Hinojosa

Málaga, 10 marzo 2017

EVALUATION COMMITTEE

President

Dr. Rafael Rivilla Palma

Departamento de Biología

Universidad Autónoma de Madrid

Secretary

Dra. Emilia Antonia López Solanilla

Centro de Biotecnología y Genómica de Plantas

Universidad Politécnica de Madrid

Vocal

Dra. Sandra de Weert

Process Development Microbiology

Koppert Biological Systems

(The Netherlands)

Substitutes

Dr. Cayo Juan Ramos Rodríaguez

Departamento de Genética

Universidad de Málaga

Dr. Jesús Mercado Blanco

Departamento Protección de Cultivos

Instituto de Agricultura Sostenible (IAS-CSIC)

Dra. Anastasia Lagopodi

Department of Crop Production

Aristotle University of Thessaloniki

(Greece)

Departamento de Microbiología

Facultad de Ciencias

ANALYSIS OF THE MICROBIAL COMMUNITY OF A SUPPRESSIVE SOIL FROM AN AVOCADO CROP

Memoria presentada por

Dña. Carmen Mª Vida Hinojosa

Para optar al grado de Doctor por la Universidad de Málaga con mención Europea

UNIVERSIDAD DE MÁLAGA

Departamento de Microbiología

Facultad de Ciencias

D. JUAN JOSÉ BORREGO GARCÍA, Director del Departamento de Microbiología de la Universidad de Málaga.

INFORMA:

Que, Dña. **CARMEN Mª VIDA HINOJOSA** ha realizado en los laboratorios de este departamento el trabajo experimental conducente a la elaboración de la presente Memoria de Tesis Doctoral.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, expedimos el presente informe,

En Málaga, a 20 de octubre del 2016

Fdo. D. Juan José Borrego García

UNIVERSIDAD DE MÁLAGA

Departamento de Microbiología

Facultad de Ciencias

D. ANTONIO DE VICENTE MORENO, Catedrático del Departamento de Microbiología de la Universidad de Málaga y

D. FRANCISCO M. CAZORLA LÓPEZ, Profesor Titular del Departamento de Microbiología de la Universidad de Málaga.

INFORMA:

Que, Dña. **CARMEN Mª VIDA HINOJOSA** ha realizado bajo su dirección el trabajo experimental conducente a la elaboración de la presente Memoria de Tesis Doctoral.

Y para que así conste, y tenga los efectos que correspondan, en cumplimiento de la legislación vigente, expide el presente informe,

En Málaga, 20 de octubre del 2016

Fdo. D. Antonio de Vicente Moreno

Fdo. D. Francisco M. Cazorla López

The present work was supported by the following funding sources:

Plan Nacional de I+D+I. Project code: AGL2011-30354-C02-01.
Title: "Estudio del control biológico de *Rosellinia necatrix* mediante el empleo de estrategias genómicas a la interacción en la rizosfera del aguacate". Principal Investigator: Dr. Francisco M. Cazorla López.

Plan Nacional de I+D+I. Project code: AGL2014-52518-C2-1-R.
Title: "Aprendiendo de las interacciones multitroficas en la rizosfera de aguacate para avanzar en el control biológico contra *Rosellinia necatrix*". Principal Investigator: Dr. Francisco M. Cazorla López.

Dña. Carmen Mª Vida Hinojosa was supported by the following grant:

Grant for "Personal investigador en formación (FPI)" from Ministerio de Economía y Competitividad (2012-2016).

Grant from Ministerio de Economía y Competitividad for a short stay in Graz University of Technology in the Institute of environmental Biotechnology (2015).

AGRADECIMIENTOS

GRACIAS a todas aquellas personas que en algún momento se cruzaron en mi camino y me ayudaron a disfrutar de esta aventura llamada tesis.

En especial me gustaría dar las gracias a mis directores de tesis, Antonio de Vicente y Francisco Cazorla. Gracias por confiar en mí desde el principio y dejarme libertad para crecer como investigadora. Gracias Antonio, por tus grandes consejos que siempre me hacen mantener los pies en la tierra, y gracias Francis, por aguantarme e inspirarme durante estos años de duro trabajo. Entre los dos habéis conseguido que aprenda a equilibrar mis decisiones. GRACIAS.

Me gustaría agradecer al resto de Profesores e Investigadores que forman parte de este gran grupo, y que siempre están dispuestos a dar su opinión y consejo. Gracias Alejandro, JC Codina, JA Torés, Diego y Cayo. Ha sido un verdadero placer escucharos y aprender de vuestra experiencia.

Gracias a todos los miembros del Departamento de Microbiología por mantener un ambiente de trabajo tan agradable. Especialmente, me gustaría agraceder al departamento la posibilidad de iniciarme en la docencia. Gracias a todos los profesores que me han ayudado en esa labor durante estos años.

A la Estación Experimental “La Mayora”, en especial a José María, Emilio y Jorge, gracias por la ayuda prestada para poder llevar a cabo ensayos incluidos en este trabajo.

MUCHÍSIMAS GRACIAS A TODOS MIS COMPAÑEROS DE LABORATORIO. SIN VOSOTROS ESTOS AÑOS NO HABRÍAN SIDO TAN ESPECIALES. Ya os he dicho que no me voy a parar a escribir todo lo que os tengo que agradecer, sonaría como una despedida, y no me apetece. Mejor seguimos compartiendo buenos ratos y creando momentos para el recuerdo (espero que durante muchos años más!!!).

A Cristian, gracias por ser mi gran apoyo, mi compañero, mi amigo…mi todo. Gracias por hacerme tan feliz y acompañarme pacientemente en este camino. TQ.

Gracias infinitas a mis padres, Manuel y Carmen, por ayudarme con vuestro esfuerzo y ánimo. Gracias por creer en mí y no permitirme abandonar mi vocación. Vuestro apoyo es mi motor y sin él nunca hubiese llegado hasta aquí. A mi hermana, Rosario, siempre lejos pero siempre juntas. Os quiero muchísimo.

A mi familia,

A Tristian,

GENERAL INDEX

FIGURES INDEX

TABLES INDEX

ABBREVIATIONS AND ACRONYMS LIST

AS: soil amended with composted almond shells

CT: soil under conventional management

WRR: white root rot

PGP: plant growth promotion

QS: *quorum sensing*

QQ: *quorum quenching*

HCN: hydrogen cyanide

PCA: phenazine-1-carboxylic acid

HPR: 2-hexyl 5-propyl resorcinol

PRN: pyrrolnitrin

PLT: pyoluteorin

DAPG: 2,4-diacetylphloroglucinol

AHLs: acyl homoserine lactones

VOCs: volatile organic compounds

ACC: 1-aminocyclopropane-1-carboxylate

ISR: induction of local and systemic resistance

ARISA: automated ribosomal intergenic spacer analysis

TRFLP: terminal restriction fragment polymorphism

DGGE: denaturing gradient gel electrophoresis

NGS: next generation sequencing

FGAs: functional gene arrays

EggNOG: public resource of orthologous groups of genes

POGs: pan-genome orthologous groups

CL/SM: confocal laser scanning microscopy

GC/MS: gas chromatography-mass spectrophotometry

BCAs: biological control agents

PCL1601: *Pseudomonas chlororaphis* PCL1601

PCL1606*: Pseudomonas chlororaphis* PCL1606

AVO110: *Pseudomonas pseudoalcaligenes* AVO110

PCL1608: *Bacillus subtilis* PCL1608

CV026: *Chromobacterium violaceum* CV026

SUMMARY

In this work, we focused in getting insight in the knowledge of soil Microbial Ecology of a suppressiveness-induced agricultural soil from an avocado (*Persea americana* Mill) crop after the application of composted almond shells as organic mulch. The study of the microbial interactions with their surroundings is essential in order to understand their involvement in plant growth and its general role in the global agricultural environment. It has been demonstrated that soil microbial community have a crucial role in the correct performance of biogeochemical cycling of nutrients, organic matter and also in the improvement of plant performance and soil quality, key issues for agroecosystem self-sustainability (Bulluck *et al.*, 2002). In order to promote and maintain the soil qualities in agriculture, farmers have used different crop management practices along years based on ecological principles. Many of these practices are used in order to control plant diseases and improve crop yield, and include crop rotation, mulches incorporated as green manures, minimal tillage practices, soil solarization and/or applications of external organic inputs (Hadar and Papadopoulou, 2012). One of these practices is the application of soil organic amendments, which has been described as safe-environmental technique, used world-wide due to the positive effects performed in different agricultural crops (Bulluck *et al.*, 2002). These positive effects on plants and soils have been associated with the maintenance of desirable soil properties, including physicochemical and microbial characteristics, such as soil aeration, structure, drainage, moisture, holding capacity, nutrient availability and microbial ecology, that often have been directly correlated with soil suppressive phenotype against different soilborne diseases (Bailey and Lazarovits, 2003). Thus, the incidence of different plant diseases caused by soilborne pathogen, could be controlled and/or reduced by the use of organic amendments from different sources (Liu *et al.*, 2007; Mendes *et al.*, 2011; Bonilla *et al.*, 2012a; Pane *et al.*, 2013; Wallisch *et al.*, 2014). The generalized use of mulches in organic management of woody perennial crops, such as avocado, could be essential due to this amendment provides

several environmental and agronomical advantages, as a C source available and long lasting, improvement in top soil layer structure, and it is a low cost material (López e tal., 2014). Moreover, the presence of this type of decomposing litter layer in the top soil surface helps to proliferate feeder roots of avocado and reduce weeds growth, allowing an improvement in plant health and yield (Wolstenholme *et al.*, 1997). One of this organic amendments is the composted almond shells (AS), which have been used to induce suppressive activity in avocado agricultural soils, leading to prevention and control of the avocado white root rot disease, causes by the soilborne phytophathogenic fungus *Rosellinia necatrix* Prill.

The application of composted almond shells as organic amendments to the avocado crop soil, influences its physicochemical soil properties, such as ions concentration of Ca^{2+}, K^{+}, Mg^{2+} and Mn^{+}, higher in soils amended with organic matter and sometimes correlated with its biocontrol ability (Bulluck *et al.*, 2002), specially when linking this activity with the presence of specific C substrates and carbon:nitrogen (C:N) ratios (Hadar and Papadopoulou, 2012). Physicochemical analysis of amended soils with composted almond shells resulted in a high content of different cations (Na^{+}, $K,^{+}$ Ca^{2+}, Mg^{2}), organic matter, C:N ratio and some micronutrients, such as Fe, Cu, Mn and Zn, which could have an influence in the growth rate of different groups of microorganisms potentially implicated in the suppressive phenotype of this soil (Gupta *et al.*, 2008).

Previous studies showed that application of composted almond shells lead to changes in soil microbial properties (Bonilla *et al.*, 2012a). Moreover, the role of microbial communities in soil suppressiveness had been widely described along years (Weller *et al.*, 2002; Haas and Defágo, 2005; Mendes *et al.*, 2011; Pane *et al.*, 2013; Bonilla *et al.*, 2015). For this reason, suppressiveness assays were performed in order to analyse the implication of soil microbiome of the agricultural avocado soil amended with composted almond shells in the biological control of the soilborne pathogen *R.*

necatrix. We used different soils from an experimental avocado field with adult avocado trees under two types of management: avocado trees only amended with composted almond shells and other group of trees under conventional management. The two types of soils were assayed using two types of experimental plant-pathogen systems, avocado and wheat (*Triticum aestivum* L.). The results showed that the soil mulched with composted almond shells displayed a better suppressive ability than conventional soil samples with a disease index significantly lower. The suppressive ability was reduced in amended almond shells soil samples when a moist-heat treatment was applied, and simultaneously, microbial population density decreased. However, no significant changes in suppressiveness were observed when the moist-heat treatment was applied to conventional soil and remains disease-conducive. The suppressive phenotype was recovered when the heat-treated soils were complemented with amended soil in 9:1 (treated:untreated; weight:weight), revealing the essential role of the microbial community present in the soil influenced by the composted almond shells in suppressiveness against *R. necatrix* in both experimental plant model used. In particular, we observed that the microbiota induced in soils amended with composted almond shells resulted crucial for the suppressiveness (Weller *et al.*, 2002). Similar studies have demonstrated this crucial role of the soil microbiome in suppressiveness, reducing the bacterial levels by using soil sterilization, autoclaving, steam pasteurization and irradiation, resulting those treated soils in conducive soils to the pathogen studied, allowing the advance of the disease (Malajczuk, 1983; Mendes *et al.*, 2010; Weller *et al.*, 2002).

Once proved the crucial key of the microbial community in suppressiveness against *R. necatrix*, we performed massive DNA sequencing assays from the amended and unamended soil samples, in order to know the microbial community potentially involved in the induced suppressiveness of this amended soil. For this purpose, we performed independent sequencing analysis of 16S ribosomic RNA gene in order to

unravel the prokaryotic community, present on the amended soil and compared with those in the unamended soils. The obtained results of the microbial community present in the amended soil, showed an increase in the relative abundance of phylum *Proteobacteria*, specially a clear increase in relative abundance of *Gamma-* and *Betaproteobacteria*. *Gammaproteobacteria* is a class of *Proteobacteria* containing bacterial representatives very well known for their plant protection abilities and their fungal interactions in previously studied suppressive soils (Mendes *et al.*, 2011; Koyama *et al.*, 2014). They included different fast-growing and easily cultivable genera from families such as *Xanthomonadaceae*, *Enterobacteriaceae* and *Pseudomonadaceae*. Nevertheless, special representation in this amended soil have the genus *Steroidobacter*, previously reported as a biodegrading bacteria. Only a few species for this genus are currently described, all of them isolated from soils with a high concentration of decomposing organic matter (Sakai *et al.*, 2014; Gong *et al.*, 2015) and involved in positive interactions with plants (Zarraonaindia *et al.*, 2015).

Simultaneously, sequencing analysis of internal transcribed spacer in ribosomal coding DNA (ITS regions) were performed in order to know the eukaryotic profile present in the amended soils. The results showed the importance of the fungal community, concretely an increase in the relative abundance of phylum *Ascomycota* was observed in the amended soil. One class of this phylum, *Dothydeomycetes* showed to be clearly increased in its relative abundance under the effect of the composted almond shells. This group of fungi have been previously reported to be abundantly present in soils with high hydrocarbon concentrations (Ferrari *et al.*, 2011). Moreover, some genera belonging to this fungal class were reported in other suppressive soils able to harbour diverse endohyphal *Gamma-* and *Betaproteobacteria*. Special importance had the order *Pleosporales*, fungi commonly isolated from plants environment (Shen *et al.*, 2014) and directly involved in the degradation of lignin, which is considered the first step of biomass conversion of plant organic matter in soils

(Ortíz-Bermúdez *et al.*, 2007). Interestingly, we observed in the soils under the influence of composted almond shells amendment, a reduction of the relative abundance of *Xylariales* order, which *R. necatrix* belong, thus revealing an antifungal effect on this group.

In order to get insight on the functional profile of this suppressive microbial community present in amended soils, we used GeoChip®, a commercial microarray, that allowed the detection of hundreds of functional microbial genes involved in different soil process such as biogeochemical cycles, environmental adaptability and plant and microorganisms interactions (Tu *et al.*, 2014). As expected, microbiome from samples of amended soil had higher hybridization to probes for C degradation (carbon cycle) related genes ranged from labile C to more recalcitrant C (e.g., starch, hemicelluloses, cellulose, chitin and lignin). These results suggest the important role of carbon degradation in the corresponding activities of the microbial community evolved in this soil. Other previous studies have observed this fact in other suppressive soils. For example, there is enough evidence to suggest a clear link between the abundance of chitins and chitin-derived C compounds in certain composts and the potential proliferation of chitinolytic microbial agents with the ability to degrade the fungal pathogen cell walls (Cretoiu *et al.*, 2013).

This functional analysis, also allowed us to analyse the specific putative activities from this amended soil. GeoChip® analysis showed that approximately 10% of the total probes analysed were unique for AS-amended samples (n= 2766 probes). When the sequence of these unique probes were analysed, we found, for example, genes for antibiotics biosynthesis such as phenazine (from *Proteobacteria*) an others related with some of the bacterial groups enhanced in the amended soil. These results suggest that a "microbe-specific suppressiveness" could been taking place in AS-amended soil. However, not only one specific group of microorganisms could be responsible of

biocontrol activity, but also multiple interactions between few groups of fungal and bacterial strains could finally result in the elicitation of suppressiveness in this soil.

Thus, agricultural avocado soil amendment with composted almond shells, promoted the selection of several specific groups of microorganisms, stimulated by the efficient and sequential use of the compounds present in almond shells. This amendment is rich in lignin (36%; López *et al.,* 2014) and lignin-degrading fungi such as *Dothideomycetes* can promote their growth, especially species from *Pleosporales* order. From lignin degradation, different aromatic compounds and C sources would be released, and they could be used then by fast-growing microorganisms such as *Gammaproteobacteria,* and *Betaproteobacteria,* where we can found the group of *Steroidobacter* spp. and other species with ability to use aromatic compounds, such as *Pseudomonas* spp., *Serratia* spp. and *Burkholderia* spp. Futhermore, these strains also produced a collection of exoenzymatic proteins (chitinases, proteases, etc), siderophores and some antifungal compounds with can lead to suppressiveness of some other group of microbes (Gross and Loper, 2009; Raaijmakers and Mazzola, 2012). In this case, after the amendment of composted almond shells, fungal order *Xylariales,* in which *R. necatrix* is included, was decreased. These results suggest, a suppressive effect against this avocado pathogen by the microbiome evolved after AS-amendment.

Because of the proposed key role of these specific members of *Gammaproteobacteria,* in this suppressive-induced soil, and due to our interest in bacterial biological control agents against *R. necatrix,* isolation and characterization of culturable members of this bacterial group, increased into the amended soil, were performed. For this purpose, we have used a selective medium described for the isolation of fluorescent Pseudomonads (Sands and Rovira, 1970). Finally, our results confirmed that this medium allowed selection of Pseudomonads and other Gram-negative bacteria from related groups. A collection of 246 Gram-negative bacteria were isolated and grouped according to their

metabolic patterns of glucose and other characteristics: *Enterobacteriaceae*-like (n= 148), fluorescent *Pseudomonadaceae*-like (n=26), nonfluorescent *Pseudomonadaceae*-like (n=11), *Xanthomonadaceae*-like (n=12) and 49 remained as unidentified isolates. Several approaches were carried out to characterize microbial activities potentially related with biological control of the diseases, including fungal antagonism, production of antimicrobial compounds and lytic exoenzymes or plant-growth-promoting (PGP) related activities. Partial sequencing of the 16S rDNA were performed to help in the isolates characterization in order to futher select some representatives to be tested on biocontrol assays, and check their potential use as *R.necatrix*-biocontrol agents (BCAs).

Antagonism was performed by the dual plate assays testing the antagonistic activity of the bacterial isolates against to 3 different soilborne fungal and oomycete pathogens, such as *R. necatrix* and *Phytophthora cinnamomi* (as avocado pathogens) and *Fusarium oxymporum* f. sp. *radicis-lycopersici* (as tomato fungal pathogen used as comparative model). The results showed that 22% of the bacterial isolates tested had antagonistic activity at least to one of the pathogens. At same time, we used colony blot assays to test the presence of biosynthetic genes of antimicrobial compounds production for the bacterial collection, such as, phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT), 2-hexyl 5-propyl resorcinol (HPR) and hydrogen cyanide (HCN) (Cazorla *et al.*, 2006; Chin-A-Woeng *et al.*, 1998; Castric, 1975; Howell and Stipanovic, 1979). These analyses showed that 11% of isolates could produce at least one of the antimicrobial compounds tested. Nevertheless, any of the 246 isolates analyzed produced DAPG or PRN. Its absence could due to that the production of these antimicrobial compounds have been described in strains with biocontrol activity only in herbaceous plant models (Hammer *et al.*, 1997; de Souza *et al.*, 2003; Barahona *et al.*,2010; Bankhead *et al.*, 2016) but not from woody plants. Additionally, production of lytic exoenzymes such as lipases,

proteases, amilases, cellulases, β-glucanases and chitinases were tested. Seventy eight percent of the isolates showed lipases, proteases and/or chitinases activities being very often detected in those soil isolates. Amilases, cellulases and β-glucanases activities were not detected in any assayed strain.

Regarding plant-growth-promotion (PGP) related activities, we analyzed both the ability of the isolates to degrade an insoluble P source, and the production of siderophores. Fifty five percent of the isolates showed at least one of the activities. At this point, and to get insight into these PGP activities, we selected 24 isolates representatives of all the diversity of results obtained, based in their characteristics evaluated in this first screening to perform *in vivo* PGP assays on tomato seedlings, showing that only 2 of the isolates showed such PGP activity.

The overall results showed that *Enterobacteriaceae*-like group were mainly producers of lytic exoenzymes and PGP-related activities whereas putative antifungal producers were mainly allocated into the *Pseudomonadaceae*-like groups, although these results could be influenced by the types of antifungal compounds assayed, more of them described as products of *Pseudomonas* spp. and related groups. Moreover, partial sequencing of the 16S rDNA of 24 selected isolates allowed the identification of 9 of these strains, all of them from *Serratia, Pseudomonas* and *Stenotrophomonas* genera.

In this point, a second selection step was performed based in the characteristics of isolates in order to perform biocontrol assays against *R. necatrix* using avocado as a susceptible plant-pathogen system (8 selected isolates). All of these strains showed biocontrol activity in avocado roots. These results confirmed that different representatives from *Gammaproteobacteria* class, after the increase of their relative abundance in suppressive amended soils, could perform a biological control activity against *R. necatrix*.

The suggested specific suppression of AS-amended soil that could be caused by various groups of microorganisms, mainly culturable Pseudomonads, had led us to

model an artificial microbial consortium of *Pseudomonas* spp. composed by *P. chlororaphis* PCL1601 and PCL1606 and *P. pseudoalcaligenes* AVO110. All of them were isolated from rhizospheres of healthy avocado trees and all of them previously screened for their antagonistic and biocontrol activity against *R. necatrix*. This ability was related with their different modes of action. *Pseudomonas chlororaphis* PCL1601 and PCL1606 are able to produce different antimicrobial compounds with antifungal activity (Cazorla *et al.*, 2006), whereas *P. pseudoalcaligenes* AVO110 is an efficiently colonizer of the avocado roots, competing preferentially for *R. necatrix* penetration sites (Pliego *et al.*, 2007).

Several previous studies have reported the different functional phenotypes of these BCAs, providing us a wide background of information very useful to gain insight in modelling the artificial consortium (Cazorla *et al.*, 2006, Pliego *et al.*, 2007; Calderón *et al.*, 2013, 2014). A deep understanding of the interactions taking place during the biocontrol process could be essential in order to improve in soil Microbial Ecology knowledge and its biotechnological implications. Microorganisms usually interact with each other (either within their own species, or across interspecies) via two main mechanisms: the contact-based interaction via physical cell-to-cell contact interchanging biomolecules and the contact-independent interaction by diffusible chemicals compounds (Song *et al.*, 2014). In order to evaluate the putative interaction among the three *Pseudomonas* sp. used in this study, we performed plate compatibility assays. The plate compatibility assays using the strain *Bacillus subtilis* PCL1608 as control bacteria, and observed that the three *Pseudomonas* sp. strains were compatible among them and inhibit PCL1608 growth. This imcompatibility between *Gammaproteobacteria* and *Firmicutes*, could explain the low relative abundance of Gram-positive strains previously observed in the suppressive amended soil. Additionally, we performed assays to detect homoserine lactones (HSLs) production, using a bioassay with the strain *Chromobaterium violaceum* CV026 as biosensor

(McClean *et al.*, 1997). Homoserine lactones are autoinducer molecules of *quorum sensing* (QS), implicated in the regulation of gene expression and population density (Parsed and Greenberg, 2000). In this case, the results shown that only PCL1606 can promote the production of violacein for CV026, by production of HSLs. This production is also detected by the bacterial consortium, suggesting that QS regulation could take place in this artificial community, but also not affecte by *quorum quenching* (QQ) regulation.

Additionally, we labelled them with different fluorescent proteins, in order to visualize the colonization patterns and spatial organization of the microbial consortium in roots of model plants, wheat and avocado. For this purpose, we used confocal laser scanning microscopy (CL/SM) technology, observing different distribution profiles including scattered single cells and mixed networks of cells covering the root surface, using both avocado and wheat roots test systems. Mainly, single cells and microcolonies were observed along roots when *Pseudomonas* sp. strains were inoculated individually. However, when the three strains were co-inoculated, mixed networks or macrocolonies without any characteristic pattern were observed, suggesting a direct, balanced and stable interaction among them. Additionally, bacterial counts showed the better stability of *Pseudomonas* sp. strains as micro- or macrocolonies, in avocado roots along time, showing a putative specialization for this niche. These results were observed independently of the presence or absence of *R. necatrix*, showing the close relationship of these rhizobacterias with root plants, especially with the avocado roots, and their ability to compete for the niche with the pathogen (Calderón *et al.*, 2014; Pliego *et al.*, 2008).

The compatible consortium of the *Pseudomonas* spp. strains used, retained the biocontrol activity, showing the ability of the bacterial consortium to reduce significantly the disease index of avocado white root rot comparing with control plants. Nevertheless, in biocontrol assays using wheat as model plant, the disease index was

not reduced significantly by Pseudomonads community, suggesting a plant specific interaction (Calderón *et al.*, 2014; Pliego *et al.*, 2008).

To gain insight in the analysis of the cooperative or cooperation behaviour of a microbial community, we performed a comparative genome analysis of the three selected Pseudomonads. For this purpose, in this study we sequenced the complete genome of *P. chlororaphis* PCL1601 and compare it with sequenced genomes of *P. chlororaphis* PCL1606 and *P. pseudoalcaligenes* AVO110. Similar genome size and GC content were observed between PCL1601 and PCL1606 (both shared the same taxonomic identification as *P. chlororaphis*), whereas AVO110 genome size was shorter and GC content higher. *Pseudomonas chlororaphis* PCL1606 has a high number of genes (number of coding sequences, CDS=5923), followed by PCL1601 (CDS=6107) and AVO110 (CDS=4475). Distribution of hierarchical clusters of orthologous genes in each of the genomes using eggNOG categories, showed a high functional homology in bacteria genomes, probably due to these strains share the ecological niche in which they inhabit and the same taxonomic classification. The analysis of clustered orthologous genes between three genomes (POGs, pan-genome orthologous groups) showed that a 36% of clusters of gene orthologous were shared by these strains, most of them from gene categories related with metabolism and cellular regulation. Moreover, this comparative analysis allowed to identify the specific genes that conferring distinctive phenotype to each specie. *Pseudomonas pseudoalcaligenes* AVO110 present specific genes related with cell motility probably due to its ability to efficiently colonize avocado roots (Pliego *et al.*, 2007). *Pseudomonas chlororaphis* PCL1601 showed a higher percentage of genes from categories related with inorganic ion transport and intracellular trafficking, suggesting an important role in environmental communication by transport of different compounds. *Pseudomonas chlororaphis* PCL1606 had specific genes involved in

DNA reparation and damage prevention, suggesting a higher adaptability to different types of stress.

In general, among others, *Pseudomonas* spp. strains are considered biological control agents due to their capacity to produce a wide range of secondary metabolites (Gross and Loper, 2009). The *in silico* analysis of the secondary metabolites production of these strains showed the potential ability of *P. chlororaphis* PCL1601 to produce phenazines, as confirmed previously (Cazorla *et al.*, 2006). These antibiotics are described in different studies to have an impact on the behavior of bacteria in the environment, acting as cell signals in QS events (Pierson and Pierson, 2010). Prediction of secondary metabolites produces by this strain showed that PCL1601 putatively produce a phenazine-derivatives associated with an ATP-binding cassette (ABC) transporter. In this case, phenazine production associated with an ABC transporter system possibly facilitated its role in cell-to-cell signaling and/or increasing antibiotics effectivity (Dietrich *et al.*, 2006). Other secondary metabolites could be produces by PCL1601, such as pyoverdine, siderophores those production could also be regulated by quorum sensing (Stintzi *et al.*, 1998).

Analysis of the putative secondary metabolites produces by *P. chlororaphis* PCL1606 detected the potential production of pyoverdine and antibiotics pyrrolnitrin (PRN) and 2-hexyl 5-propyl resorcinol (HPR), as previously reported (Cazorla *et al.*, 2006; Calderón *et al.*, 2015). In genome of this strain we also found a cluster related with the production of homoserine lactones (HSLs), suggesting its ability to regulate some phenotypes by QS (Parsed and Greenberg, 2000) and HSLs production has been confirmed in this work.

The analysis of secondary metabolites potentially produced by *P. pseudoalcaligenes* AVO110, revealed that any potential cluster identified had a percentage of similarity higher than 58%. A putative polyketide synthetase type I (58% similarity) was detected, which belong to a large class of natural products with a vast array of

antimicrobial activities (Chan *et al.*, 2009). Nevertheless, no siderophores or antibiotics very well-known were detected, results supported by previous studies (Pliego *et al.*, 2007).

All of these previous compounds could be implicated in fungal antagonistic activities and microbial interactions. Nevertheless, bacteria could produce other types of compounds involved in these purposes. Recent studies demonstrated the key role of bacterial volatiles such as infochemicals molecules in microbial communication. Some of the bacterial metabolized compounds can be emitted as volatile products that are readily used by other organisms. The volatiles compounds can be of organic or inorganic nature and many functions could be attributed them: playing a role in the food chain, influence physiological processes, playing a role in QS, acting as antimicrobial compounds and could be used as community signals (Effmert *et al.*, 2012). Previous studies demonstrated that *P.chlororaphis* PCL1601 and PCL1606 produced the inorganic volatile compound, HCN widely described in the *Pseudomonas* genus, by its antifungal activity probably due to the inhibition effect of several metal-containing enzymes, under *quorum sensing* regulation (Effmert *et al.*, 2012). However, nothing is known about *P. pseudoalcaligenes* AVO110 volatiles production.

By dual divided plate assays, effect of the volatiles production by single strains and microbial consortium in the antagonistic activity against *R. necatrix* was evaluated. A significant reduction of fungal growth was observed in all conditions tested, with a highest reduction of fungal mycelium produced by the microbial consortium. In order to get insight into the production of volatiles by these bacterial strains, detection and characterization of volatiles organic compounds (VOCs) were performed using GC/MS-based (gas chromatography/mass spectrometry) headspace approaches. These experiment, using individual inoculations showed that *P. pseudalcaligenes* AVO110 efflux a high number of VOCs (n= 13) than *P. chlororaphis* strains (PCL1601, n= 5;

PCL1606, n= 8). But when the strains were inoculated combined (in the same proportion, in the same plate) a higher number of VOCs were produced (n= 15). Thus, the two compounds produced in greater quantity by microbial consortium were dimethyl disulfide and 1-undecene. These compounds have been described as VOCs typically produced by the *Pseudomonas* genus, and related groups with ability to suppress growth of different organisms, concretely, fungal mycelium and spores (Popova *et al.*, 2014; Fernando *et al.*, 2005). Moreover, 1-undecene produced by *Pseudomonas* spp., have been also described to inhibit growth capacity of some oomycete such as *Phytophthora infestans* (Hunziker *et al.*, 2015). Interestingly, we detected 3 VOCs only produced by the microbial consortium, but not by the individual strains. Two of these volatiles were the hydrocarbons pentadecane and heptadecane, with an unknown function. Their production by microbial consortium, but not by single strains, could suggest the use of co-metabolism among the three Pseudomonads strains. The third compound detected was S-methyl 3-methylbutanethioate, for which also nothing is known about its function in literature. Nevertheless, a similar compound, S-methyl butanethioate, is a volatile compound produced by different *Pseudomonas* sp. strains and with a specific effect on inhibition of sporangia germination, mycelial growth and zoospore motility of oomycetes such as *Phytophthora infestans* (Vrieze *et al.*, 2015).

This approximation analysis of the putative interactions that take place during biological control of *R. necatrix* by an artificial microbial consortium of Pseudomonads, could open the doors to design targeted future experiments of transcriptomics, proteomics and/or metabolomics techniques, in order to gain insight about the microbial ecology and the biological process that take place during biocontrol of avocado white root rot.

RESUMEN

Este estudio se ha dirigido a profundizar en diferentes aspectos relacionados con la Ecología Microbiana y los distintos procesos que tienen lugar en un suelo agrícola de un cultivo de aguacate (*Persea americana* Mill) que desarrolla una actividad supresiva tras la aplicación de una enmienda orgánica. El estudio de las interacciones que tienen lugar dentro de la comunidad microbiana de un suelo, es esencial para poder entender la implicación de los distintos microorganismos en el crecimiento vegetal y su comportamiento general del ecosistema. Dicha comunidad microbiana tiene un papel primordial en el correcto funcionamiento de los ciclos biogeoquímicos de diferentes nutrientes, en la degradación de materia orgánica y mejora de la salud vegetal y la calidad del suelo, factores muy importantes para una agricultura sostenible (Bulluck *et al.*, 2002). Existen diferentes prácticas de manejo agrícola basadas en principios ecológicos y que han sido utilizadas por los agricultores a lo largo de los años para promover y mantener la calidad del suelo. Muchas de estas técnicas se pueden utilizar para controlar enfermedades que pueden afectar al cultivo y para mejorar la producción de estos. Así, la rotación de cultivos, uso de cubiertas vegetales, uso controlado de la labranza, solarización de los suelos y/o aplicación de materia orgánica de diferentes orígenes (Hadar and Papadopoulou, 2012) son ejemplos de técnicas agrícolas utilizadas para este fin. La aplicación de enmiendas orgánicas ha sido descrita en diferentes trabajos como una técnica ambientalmente sostenible y utilizada en todo el mundo, debido al efecto positivo que generalmente causa en los cultivos (Bulluck *et al.*, 2002). Este efecto ha sido asociado al mantenimiento de las propiedades del suelo, tanto fisicoquímicas como biológicas, que frecuentemente se han relacionado directamente con la actividad supresiva de algunos suelos frente a diferentes enfermedades de plantas causadas por patógenos de suelos. Diferentes trabajos recogen como la incidencia de diferentes enfermedades de plantas causadas por patógenos de suelo puede ser controlada y/o reducida por el uso de enmiendas orgánicas de diferente naturaleza (Liu *et al.*, 2007; Mendes *et al.*, 2011; Bonilla *et al.*,

2012a; Pane *et al.*, 2013; Wallisch *et al.*, 2014). Un ejemplo de enmienda orgánica es la cáscara de almendra compostada, que induce actividad supresiva en suelos agrícolas de cultivos de aguacate, ya que previenen y controlan la podredumbre blanca radicular el aguacate, causada por el hongo fitopatogéno *Rosellinia necatrix* Prill. El uso de materia orgánica compostada, en el manejo de cultivos de leñosas de hoja perenne, como el aguacate, es una práctica esencial ya que puede conferir ventajas tanto ecológicas como agrícolas. La cáscara de almendra constituye un residuo orgánico de la industria almendrera, y que con su posible uso en agricultura, permitiría su reutilización y le aporta un nuevo valor añadido. Además, su aplicación supone la disponibilidad de una fuente de C de lenta degradación y larga duración, resistente y de bajo coste (López *et al.*, 2014). La aplicación de capas de materia orgánica en degradación en la superficie del cultivo del aguacate, es una práctica habitual en este cultivo. La presencia de dicha capa orgáncia favorece la proliferación de las raíces alimenticias del aguacate en la parte superior del suelo, así como una reducción en el crecimiento de malas hierbas, causando una mejora en la salud de la planta y por lo tanto, en la producción de cultivo (Wolstenholme *et al.*, 1997).

En nuestro trabajo, se puso de manifiesto que la aplicación de cáscara de almendra compostada como enmienda orgánica influye en diferentes factores como la concentración de iones de Ca^{2+}, K^{+}, Mg^{2+} y Mn^{+}, más abundantes en los suelos enmendados y correlacionados con la capacidad supresiva del suelo (Bulluck *et al.*, 2002). También incrementa la presencia de fuentes de C específicas y de la razón carbono:nitrógeno (C:N; Hadar and Papadopoulou, 2012), indicador general de la fertilidad del suelo. Los análisis fisicoquímicos realizados al suelo enmendado con cáscara de almendra compostada también revelaron un alto contenido en algunos micronutrientes como Fe, Cu, Mn y Zn, y que pueden influir en la tasa de crecimiento de algunos grupos de microorganismos implicados en la actividad supresiva de este suelo (Gupta *et al.*, 2008).

Ya en estudios previos se evidenció que la aplicación de la cáscara de almendra compostada también causaba cambios en las propiedades microbianas del suelo (Bonilla *et al.*, 2012a). Además, el papel de la comunidad microbiana en la supresividad del suelo ha sido ampliamente descrito a lo largo de los años en otros trabajos (Weller *et al.*, 2002; Haas and Defágo, 2005; Mendes *et al.*, 2011; Pane *et al.*, 2013; Bonilla *et al.*, 2015). Por esta razón, se realizaron ensayos de supresividad para comprobar la implicación del microbioma que se desarrolla bajo la influencia del suelo enmendado, en el control del patógeno *R. necatrix*. Para llevar a cabo estos ensayos, se emplean muestras obtenidas de una parcela experimental de cultivo de aguacate con más de 100 árboles adultos sometidos a 2 tipos de manejo diferente: uno orgánico, con la aplicación masiva de cáscara de almendra compostada desde hace años, y otro sometido a un manejo convencional con los productos químicos recomendados. Además, en el laboratorio se emplearon dos tipos de modelos experimentales de plantas susceptibles al hongo fitopatógeno *R. necatrix*, aguacate y trigo (*Triticum aestivum* L.). Estos ensayos de supresividad mostraron que los suelos enmendados con cáscara de almendra tenían actividad supresiva frente a *R. necatrix*, mientras que los suelos de manejo convencional permanecían conductivos, con valores de índice de enfermedad significativamente mayores que los suelos enmendados. Por otro lado, la supresividad se veía reducida en las muestras de suelos enmendados tras la aplicación de un tratamiento térmico, que reducía la densidad microbiana. Además, observamos la recuperación del fenotipo supresivo de un suelo, cuando se complementaban con suelo enmendado original en una proporción 9:1 (tratado:no tratada; peso:peso). Estos resultados mostraron el importante papel de la comunidad microbiana de los suelos enmendados con cáscara de almendra compostada en la supresividad frente a *R. necatrix*, y que se puso de manifiesto empleando ambos modelos de planta ensayados. La recuperación de la supresividad cuando se complementan los suelos tratados térmicamente con una parte de suelo enmendado, indica una supresividad influenciada

por la actividad de grupos específicos de microorganimos que se desarrollan en el suelo enmendado (Weller *et al.*, 2002). Nuestros resultados confirman lo ya observado en trabajos previos con otros sistemas, donde demuestran que el papel del microbioma del suelo puede tener en la supresividad, ya que tras tratamientos que reducen el número de microorganismos al suelo supresivo como la esterilización, pasteurización o irradiación, se conseguía perder la actividad supresiva de los suelos, favoreciendo así, el avance de la enfermedad (Malajczuk, 1983; Weller *et al.*, 2002; Mendes *et al.*, 2011). En todos los casos el papel de la microbiota del suelo era esencial para el control de la enfermedad, y se consideraban los dos tipos clásicos de supresividad descritos: una supresividad general dependiente de la comunidad microbiana completa y no transferible a otros suelos o una supresividad específica dependiente de algunos grupos de microorganismos y transferible a otros suelos (Weller *et al.*, 2002).

Una vez comprobado el importante papel de la comunidad microbiana en la supresividad del suelo enmendado con cáscara de almendra compostada contra *R. necatrix*, se inició una estrategia de análisis metagenómico para conocer los grupos microbianos presentes en el suelo. Para ello, llevamos a cabo la secuenciación masiva del ADN total de distintas muestras de suelo comparando suelo enmendado con suelo bajo manejo convencional, para conocer sus perfiles microbianos. Se realizaron secuenciaciones independientes del gen del ARN ribosómico de 16S para conocer el perfil procariótico de la comunidad microbiana. Los resultados mostraron el incremento en la abundancia relativa del phylum *Proteobacteria*, en concreto, el aumento de la abundancia relativa de las clases *Gamma-* y *Betaproteobacteria.* Hay que destacar que, *Gammaproteobacteria* es una clase de *Proteobacteria* ampliamente descritas en otros suelos supresivos por la capacidad de algunos de sus representantes para estimular la protección vegetal o para interaccionar contra patógenos fúngicos (Mendes *et al.*, 2011; Koyama *et al.*, 2014). En este grupo podemos encontrar diferentes grupos de bacterias cultivables en medios artificiales, de fácil manejo y

crecimiento rápido, como por ejemplo representantes de las familias *Xanthomonadaceae, Enterobacteriaceae* y *Pseudomonadaceae*. No obstante, destaca en el suelo modificado por la aplicación de cáscara de almendra compostada, la presencia el género *Steroidobacter* el cual tiene un interés especial como bacterias directamente implicadas en la degradación del material orgánico. Muy pocas especies de este género están descritas en la actividad, y algunas de ellas aisladas de suelos con una alta concentración en materia orgánica en descomposición (Sakai *et al.*, 2014; Gong *et al.*, 2015) e involucradas en interacciones beneficiosas con plantas (Zarraonaindia *et al.*, 2015), lo que sugiere un papel relevante dentro de la microbiota que se desarrolla en los suelos enmendados con cáscara de almendra.

Paralelamente, se analizaron las secuencias de las regiones intergénicas (ITS) del ARN ribosómico 18S, para conocer el perfil eucariótico del microbioma del suelo enmendado. Los resultados muestran la importancia de la comunidad fúngica en estos suelos. En concreto, se observó el incremento en la abundancia relativa del phylum *Ascomycota*, principalmente de la clase *Dothideomycetes* en las muestras de suelo enmendado. La abundancia de este grupo de hongos ha sido descrita anteriormente en suelos con altas concentraciones de compuetos hidrocarbonados (Ferrari *et al.*, 2011). Se ha descrito la presencia de algunos representantes de esta clase en suelos supresivos, que además tendrían capacidad de portar especies de *Gamma-* y *Betaproteobacteria* como bacterias endohifales. Dentro de este grupo, mostró especial interés en este suelo el orden *Pleosporales*, grupo de hongos aislados de plantas (Shen *et al.*, 2014) e implicados en los primeros pasos de degradación de la lignina (Ortíz-Bermúdez *et al.*, 2007). Este análisis del perfil eucariótico de microorganismos, además mostró la reducción de la abundancia relativa del orden *Xylariales*, grupo al que pertenece el hongo patógeno *R. necatrix*, siendo este hecho la evidencia directa de la supresividad frente a este patógeno en suelos enmendados.

Para profundizar en el perfil funcional de esta comunidad microbiana, se empleó el análisis con GeoChip®, un microarray comercial que detecta cientos de genes microbianos funcionales y potencialmente implicados en diferentes procesos que se llevan a cabo en el suelo, bien como parte de los ciclos biogeoquímicos, actividades de adaptación ambiental e interacción con plantas otros microorganismos (Tu *et al.*, 2014). En nuestro caso, las muestras de ADN extraido desde suelo enmendado, presentaron un mayor número de sondas relacionadas con la degradación de diferentes fuentes de C, desde formas más lábiles de C hasta más recalcitrantes (almidón, hemicelulosa, celulosa, quitina y lignina). Este efecto también se ha observado en otros suelos supresivos, donde las evidencias sugieren un claro vínculo entre la abundancia de quitinas y compuestos derivados en algunos compost con la proliferación de agentes microbianos quitinolíticos con actividad antifúngica (Cretoiu *et al.*, 2013).

Los resultados del análisis del GeoChip®, mostraron que aproximadamente el 10% del total de las sondas que hibridaron en el microarray solo estaban presentes en las muestras de suelos enmendados (n= 2766). Al analizar la secuencia de estas sondas específicas, se puso de manifiesto la hibridación con genes de biosíntesis de antibióticos como la fenazina (de miembros bacterianos pertenecientes al phylum *Proteobacteria*) y otros representantes pertenecientes a grupos de bacterias que habían aumentado su presencia en los suelos enmendados.

Estos resultados sugieren que la supresividad inducida por la aplicación de cáscara de almendra compostada al suelo podría tener un carácter específico, es decir, se debería a las actividades desarrolladas por algunos grupos de microorganismos concretos de este suelo. Probablemente, no se deba a la actividad de un único grupo de microorganismos sino a las interacciones que tienen lugar entre diferentes grupos de hongos y bacterias presentes en la comunidad microbiana de este suelo.

Así, el suelo enmendado con cáscara de almendra compostada promovería la selección de grupos concretos de microorganismos estimulados por su capacidad para la

degradación de este tipo de materia orgánica. Como esta enmienda orgánica es rica en lignina, algunos hongos degradadores de lignina como los *Dothideomycetes* pueden promover su crecimiento, especialmente los representantes del orden *Pleosporales*. Fruto de la degradación inicial de la lignina, se originarían diferentes compuestos aromáticos y otras fuentes de C, más accesibles para microorganismos de crecimiento rápido como son las *Gammaproteobacteria* y *Betaproteobacteria*, donde destacan las especias de *Steroidobacter* spp. con capacidad para utilizar diferentes compuestos aromáticos y *Pseudomonas* spp., *Serratia* spp. y *Burkholderia* spp. Además, se ha descrito que estas cepas portan una colección de exoenzimas (quitinasas, proteasas), sideróforos y compuestos antifúngicos, que pueden estar relacionados en la supresividad contra patógenos de estos microorganismos (Gross and Loper, 2009; Raaijmakers and Mazzola, 2012). Como resultados de todas estas interacciones, se observa una disminución en la abundancia del orden de los *Xylariales*, grupo al que pertenece la especie fitopatógena *R. necatrix* y causante de la podredumbre blanca radicular. Su disminución se puede interpretar como consecuencia del efecto supresivo del microbioma que se estimula en los suelos enmendados con cáscara de almendra contra este patógeno de aguacate.

Debido al importante papel que las *Gammaproteobacteria* tienen en este suelo y a nuestro interés en agentes bacterianos de control biológico frente a *R. necatrix*, nuestro trabajo se centró en el aislamiento y caracterización de aquellos microorganismos cultivables pertenecientes a las gammaproteobacterias, y mas concretamente al género *Pseudomonas*, y que habían mostrado ser más abundantes en los suelos enmendados. Para llevar a cabo esta tarea, se utilizó un medio selectivo descrito para el aislamiento de pseudomonas fluorescentes (Sands y Rovia, 1970) pero que también permitía el aislamiento de otros grupos de bacterias Gram-negativas relacionados. Se obtuvo una colección de 246 bacterias Gram-negativas, que fueron agrupadas según distintas caracteristicas: enterobacterias (*Enterobacteriaceae*-like, n= 148), pseudomonas

fluorescentes (fluorescent *Pseudomonadaceae*-like, n= 26), pseudomonas no fluorescentes (nonfluorescent *Pseudomonadaceae*-like, n= 11), xanthomonas (*Xanthomonadaceae*-like, n= 12) y 49 aislados que quedaron sin clasificar. Además, llevamos a cabo diferentes experimentos para caracterizar actividades microbianas relacionadas con el control biológico de enfermedades como ensayos de antagonismo, producción de compuestos antimicrobianos o exoenzimas líticas, y ensayos de actividades relacionadas con la promoción del crecimiento vegetal (PGP). La secuenciación parcial del gen del RNA ribosómico de 16S también se realizó para facilitar la caracterización de algunos aislados y facilitar la selección de algunos aislados representativos para evaluar su potencial actividad como agentes de control biológico frente a *R. necatrix.*

A continuación, se llevaron a cabo ensayos de antagonismo en placa para evaluar este fenotipo en la colección de aislados obtenidos frente a 3 patógenos diferentes: *R. necatrix* y *Phytophthora cinnamomi* (patógenos de aguacate) y *Fusarium oxysporum* f. sp. *radicis-lycopersici* (patógeno de tomate, y ampliamente usado como modelo de estudio).

Los resultados de los experimentos mostraron que el 22% de los aislados bacterianos tenían alguna actividad antagonista, al menos frente a uno de los patógenos ensayados. Al mismo tiempo, se analizó la presencia de genes potencialmente implicados en la biosíntesis de compuestos antimicrobianos antifúngicos en la colección de aislados. Para ello, se realizaron ensayos de hibridación de sondas (mediante "colony blots") para detectar la presencia de los genes biosintéticos para los compuestos fenazina-1-ácido carboxílico (PCA), 2,4-diacetilfloroglucinol (DAPG), pirrolnitrina (PRN), pioluteorina (PLT), 2-hexil 5-propil resorcinol (HPR) y ácido cianhídrico (HCN) (Castric, 1975; Howell and Stipanovic, 1979; Chin-A-Woeng *et al.*, 1998; Cazorla *et al.*, 2006). Los resultados mostraron que el 11% de los aislados podría producir al menos uno de los antimicrobianos analizados. No obstante, ninguno de los 246

aislados analizados podría producir DAPG o PRN. Este resultado está de acuerdo con las observaciones que sugieren que estos antibióticos son característicos de cepas con actividad de control biológico en modelos de plantas herbáceas (Hammer *et al.*, 1997; de Souza *et al.*, 2003; Barahona *et al.*, 2010; Bankhead *et al.*, 2016). Simultáneamente, analizamos la producción de exoenzimas líticas como las lipasas, proteasas, amilasas, celulasas, β-glucanasas y quitinasas. El 78% de los aislados presentaron actividad lipasa, proteasa y/o quitinasa, mientras que las actividades amilasa, celulasa y β-glucanasa no fueron detectadas en ningún aislado.

En cuanto a las actividades relacionadas con la promoción del crecimiento vegetal, la degradación de una fuente insoluble de fósforo, y la producción de sideróforos, el 55% de los aislados mostraron actividad en al menos una de estas pruebas.

Teniendo en cuenta todos estos resultados, se seleccionaron 24 de los aislados que representaran la mayor diversidad posible, y se llevaron a cabo ensayos de PGP *in vivo*. Tras estos ensayos, solo 2 de los aislados mostraron una actividad de promoción del crecimiento vegetal de semillas de tomate. En general, estos análisis mostraron que dentro del grupo *Enterobacteriaceae*-like se encuentra un mayor número de aislados bacterianos productores de exoenzimas líticas y promotores del crecimiento vegetal mientras que los posibles productores de antibióticos pertenecían al grupo *Pseudomonadaceae*-like, aunque este resultado puede estar influenciado por la batería de antifúngicos ensayados, la mayoría descritos en *Pseudomonas* spp. y en grupos relacionados. Además, la secuenciación parcial del gen del ARN ribosómico 16S de estos 24 aislados seleccionados, junto con los resultados obtenidos en las pruebas bioquímicas de identificación, nos permitió asignar el género al que pertenecen 9 de estas cepas, todas ellas incluidas en *Serratia* sp., *Pseudomonas* sp. y *Stenotrophomonas* sp.

Finalmente, se eligieron a 8 aislados diferentes para realizar los ensayos de control biológico frente a *R. necatrix* en aguacate en base a las características observadas

anteriormente. Todas las cepas ensayadas presentaban actividad de control biológico frente *R. necatrix*. Estos resultados apoyarían la hipótesis del papel crucial de diferentes grupos específicos de *Gammaproteobacteria* que aumentan su abundancia relativa en los suelos supresivos enmendados con cáscara de almendra, ya que los aislados estudiados presentan actividad de control biológico frente a *R. necatrix.* Teniendo en cuenta los resultados obtenidos hasta el momento sobre la supresividad específica que se desarrolla en los suelos enmendados con cáscara de almendra es debida a grupos concretos de microorganismos, y dada la actividad de control biológico demostrada por algunas de las pseudomonas previamente aisladas de estos, se decidió iniciar los estudios sobre las interacciones que se pueden llevar a cabo entre los organismos implicados en el control biológico de *R. necatrix*. El conocimiento sobre las interacciones que tienen lugar durante el proceso de control biológico podría ser esencial para mejorar en el conocimiento de la Ecología Microbiana de este tipo de suelos y en las posibles implicaciones biotecnológicas que pudieran derivarse. Los miembros de una comunidad microbiana emplean diversas herramientas moleculares para sincronizar sus respuestas. Los microorganismos normalmente interaccionan los unos con los otros (inter- o intra- específicamente) utilizando dos tipos de mecanismos: la interacción basada en el contacto físico célula-célula y la interacción independiente de contacto basada en el intercambio de compuestos difusibles (Song *et al.*, 2014). Con objeto de abordar dicho estudio desde un punto de vista más completo, y que simula una posible comunidad microbiana básica, se planteó la construcción de un consorcio microbiano artificial, como modelo de estudio empleando rizobacterias bien caracterizadas. Dicho consorcio microbiano artificial estaría formado por *Pseudomonas chlororaphis* PCL1601, *Pseudomonas chlororaphis* PCL1606 y *Pseudomonas pseudoalcaligenes* AVO110, descritas como agentes de control biológico contra *R. necatrix* en trabajos anteriores. Estas cepas fueron aisladas de la rizosfera de aguacates sanos y caracterizadas mediante diferentes experimentos. Cada

una de las cepas muestra modos de acción diferentes, que pueden ser complementarios entre si. En concreto, estas cepas mostraron actividad antagonista y de control biológico frente a *R. necatrix*. Además, se comprobó la producción de diferentes antimicrobianos por parte de las cepas de *P. chlororaphis* PCL1601 y PCL1606 (Cazorla *et al.*, 2006) y la capacidad de *P. pseudoalcaligenes* AVO110 para colonizar eficientemente las raíces de aguacate, preferentemente por los sitios de penetración del hongo (Pliego *et al.*, 2007).

Los trabajos previos han permitido la caracterización de los fenotipos funcionales de estas cepas, incluyendo entre otros, producción de antifúngicos, colonización de raíces y control biológico (Cazorla *et al.*, 2006; Pliego *et al.*, 2007; Calderón *et al.*, 2013, 2014). Para comprobar si el consorcio formado por las tres cepas de *Pseudomonas* sp. podía ser viable, se realizaron ensayos de compatibilidad en placa entre los distintos componentes. Los resultados mostraron que las tres pseudomonas no inhibían el crecimiento en placa entre sí, pero que si inhibían el crecimiento de otra cepa de control biológico utilizada, *Bacillus subtilis* PCL1608, aislada también del mismo nicho ecológico. Esta posible imcompatibiliad de crecimiento entre cepas de *Gammaproteobacteria* y *Firmicutes*, podría apoyar los resultados previamente observados sobre la baja abundancia relativa de representantes de cepas de Gram-positivas en el suelo supresivo enmendado con cáscara de almendra. Paralelamente, realizamos ensayos de producción de homoserina lactonas (HSLs) mediante bioensayos utilizando la cepa de *Chromobacterium violaceum* CV026 como biosensor (McClean *et al.*, 1997). Las HSLs son moléculas autoinductoras de *quorum sensing* (QS), implicadas en la regulación de la expresión de genes dependientes de la densidad poblacional (Parsed and Greenberg, 2000). En este caso, los resultados mostraron que solo PCL1606 era capaz de promover la producción de violaceína por parte de CV026, y por lo tanto, capaz de producir HSLs en este bioensayo. Dicha producción de HSLs, se mantiene en el consorcio microbiano, sugiriendo la posible regulación también de

diferentes procesos via QS en esta comunidad artificial, así como que ningún proceso de *quorum quenching* (QQ) estaría teniendo lugar dentro del consorcio.

Adicionalmente, marcamos las cepas con diferentes proteínas fluorescentes para confirmar su estabilidad y visualizar los patrones de colonización y organización espacial del consorcio microbiano en la raíz de las plantas modelo (trigo y aguacate). Para este propósito, se empleó la microscopía confocal de fluorescencia. Principalmente, se observó una distribución de células individuales y microcolonias a lo largo de las raíces cuando las cepas de *Pseudomonas* sp. se inocularon de forma independiente. No obstante, cuando las tres cepas fueron coinoculadas, se observaron agrupaciones mixtas de células o macrocolonias, sugiriendo una interacción equilibrada y estable entre ellas. Estos resultados se observaron independientemente de la presencia o ausencia de *R. necatrix*, sugiriendo la estrecha relación de estas rizobacterias con la raíz, especialmente por la de aguacate, y su capacidad para competir por el nicho con el patógeno (Calderón *et al.*, 2014; Pliego *et al.*, 2008). De forma adicional, los recuentos bacterianos mostraron una mejor estabilidad de las cepas de *Pseudomonas* sp. y una posible especialización por este nicho.

Una vez conocida la compatibilidad entre las cepas del consorcio artificial, se llevaron a cabo ensayos de control biológico, frente a *R necatrix*. Los resultados mostraron la capacidad de la comunidad sintética diseñada para reducir de forma significativa el índice de enfermedad en plantas de aguacate. Sin embargo, en los ensayos de control biológico realizados con trigo como planta modelo, no se observó protección frente a *R. necatrix*, probablemente debido a una posible especificidad de los miembros del consorcio por los exudados de aguacate, y/o por el nicho (Calderón *et al.*, 2014; Pliego *et al.*, 2008).

Trabajos anteriores, sugieren que el análisis de los genomas de las especies que forman una comunidad microbiana, podría ayudar a dilucidar los posibles comportamientos de cooperación o competencia que tienen lugar durante la interacción microbiana

(Mitri and Foster, 2013). Por este motivo, se realizó un análisis comparativo de los genomas de las 3 especies de *Pseudomonas* spp. Para llevar a cabo este propósito, se secuenció el genoma completo de *P. chlororaphis* PCL1601 y lo comparamos con los genomas, ya secuenciados previamente, de *P. chlororaphis* PCL1606 y *P. pseudoalcaligenes* AVO110. Se observan similitudes entre el tamaño del genoma y el contenido en GC entre PCL1601 y PCL1606 (ambas comparten la misma identificación taxonómica como *P. chlororaphis*), mientras que el genoma de AVO110 era más pequeño y el contenido en GC mayor.

El análisis de la distribución de los clusters de genes ortólogos en cada uno de los genomas utilizando las categorías eggNOG, mostró una alta homología funcional entre las cepas, probablemente debido a que comparten el mismo nicho ecológico y el mismo género. Este análisis mostró que el 36% de los clusters de genes ortólogos entre genomas estaban compartidos por las tres cepas, y que la mayoría de estos genes pertenecían a categorías relacionadas con el metabolismo y la regulación celular. Además, este tipo de análisis comparativo nos permitió identificar genes específicos que les conferían un carácter distintivo a cada una de ellas. Así, *P. pseudoalcaligenes* AVO110 presentaba genes específicos relacionados con la movilidad celular, relacionado probablemente, a su capacidad para colonizar las raíces de aguacate (Pliego *et al.*, 2007). *Pseudomonas chlororaphis* PCL1601 presentaba un porcentaje mayor de genes relacionados con categorías relacionadas con el transporte de iones inorgánicos y el tráfico intracelular, sugiriendo un papel importante en la comunicación con su entorno mediante el transporte de diferentes compuestos. *Pseudomonas chlororaphis* PCL1606 presentaba genes específicos relacionados con la reparación y la prevención de daños en el ADN, sugiriendo una mejor adaptabilidad a diferentes tipos de estrés.

Adicionalmente, el análisis *in silico* de la producción de metabolitos secundarios de estas cepas mostró la capacidad de *P. chlororaphis* PCL1601 para producir fenazinas,

producción ya descrita en trabajos anteriores (Cazorla *et al.*, 2006). Estos antibioticos están descritos por su impacto en el comportamiento de la bacteria con su entorno, actuando como molécula señal en distintos procesos (Pierson and Pierson, 2010). Otros posible metabolito secundario producido por PCL1601 fue la pioverdina, sideróforo cuya producción podría estar regulada por QS (Stintzi *et al.*, 1998).

Por otro lado, el análisis de los posibles metabolitos secundarios producidos por *P. chlororaphis* PCL1606 detectó clusters de genes de producción de pioverdina y de los antibióticos pirrolnitrina (PRN) y 2-hexil 5-propil resorcinol (HPR) cuya producción ya estaba descrita por trabajos anteriores (Cazorla *et al.*, 2006; Calderón *et al.*, 2015). En el genoma de esta cepa encontramos también un cluster de genes de producción de homoserinas lactonas (HSLs), proteínas implicadas en la regulación por QS de expresión de genes por densidad poblacional (Parsed and Greenberg, 2000) y cuya producción se ha comprobado en este trabajo en ensayos descritos anteriormente. Finalmente, el análisis de potenciales metabolitos secundarios producidos por *P. pseudoalcaligenes* AVO110, mostró que ninguno de los clusters detectados presentaba una similitud de secuencia superior al 58% con algun otro conocido, resultado descrito anteriormente (Pliego *et al.*, 2007).

Estudios recientes han demostrado el importante papel que los compuestos volátiles pueden tener como moléculas señal en la comunicación microbiana con diversas funciones: pueden tener un papel principal en las cadenas alimenticias, influir en procesos fisiológicos, actuar como moléculas señal de QS y/o actuar como antimicrobianos (Effmert *et al.*, 2012). Estudios previos demostraron que *P.chlororaphis* PCL1601 y PCL1606 producían el compuesto volátil inorgánico ácido cianhídrico (HCN; Cazorla *et al.*, 2006), ampliamente descrito por su actividad antifúngica debido al efecto inhibitorio que causa a enzimas transportadoras de metales, regulado por QS (Effmert *et al.*, 2012). Por otro lado, nada se conoce sobre la producción de volátiles por parte de la cepa *P. pseudoalcaligenes* AVO110 salvo

que no es capaz de producir HCN (Pliego *et al.*, 2007). Por esta razón, se llevaron a cabo ensayos duales en placas divididas para evaluar el efecto de la producción de compuestos volátiles orgánicos (VOC) por parte de las cepas de forma individual y del consorcio microbiano en la actividad antagonista frente a *R. necatrix*. En estos ensayos, se observó una reducción significativa del crecimiento del hongo en todas las condiciones ensayadas, siendo mayor la reducción del crecimiento fúngico causada por el consorcio microbiano. A raíz de los resultados obtenidos, y para profundizar en la producción de compuestos volátiles por las diferentes cepas y el consorcio bacteriano, se realizaron experimentos de detección y caracterización de volátiles mediante cromatografía de gases/espectrometría de masas, GC/MS. Estos experimentos nos permitieron conocer los VOC producidos en cada condición. Los resultados mostraron que *P. pseudoalcaligenes* AVO110 producía un mayor número de VOC (n= 13) en comparación con las cepas de *P. chlororaphis* (PCL1601, n= 5; PCL1606, n= 8). Cuando estas cepas se coinocularon (en la misma proporción, en la misma placa) observamos un mayor número de VOC producidos (n= 15). En ese caso, 2 de los compuestos producidos en mayor cantidad por el consorcio bacteriano fueron el dimetil disulfito y el 1-undeceno. Estos compuestos forman parte del perfil típico de VOC emitidos por el género *Pseudomonas* sp. y otros grupos relacionados. Ambos compuestos volátiles han sido identificados por su capacidad para inhibir el crecimiento de diferentes organismos, concretamente el crecimiento miceliar y de esporas de algunos hongos (Popova *et al.*, 2014; Fernando *et al.*, 2005). Además, el 1-undeceno producido por *Pseudomonas* spp. inhibe el crecimiento de oomicetes, como *Phytophthora infestans* (Hunziker *et al.*, 2015). Además de estos compuestos, detectamos la producción de 3 volátiles orgánicos que solo eran producidos por el consorcio artificial. Dos de estos volátiles eran el pentadecano y el heptadecano, con una función desconocida hasta el momento. La producción de estos VOC sólo en presencia de las tres cepas bacterianas que forman el consorcio sugiere el uso del co-

metabolismo entre las tres cepas de pseudomonas. Un tercer compuesto volátil producido solo por el consorcio microbiano fue el S-metil 3-metilbutanotioato, también de función desconocida. No obstante, un compuesto similar, el S-metil butanotioato, producido por diferentes cepas de *Pseudomonas* sp. ha sido descrito previamente como un compuesto volátil con efecto de inhibición de la germinación de esporangios, crecimiento miceliar y movilidad de zoosporas en algunos oomicetes como *Phytophthora infestans* (Vrieze *et al.*, 2015).

Así, el consorcio microbiano construido, podría utilizarse en estudios sobre las posibles interacciones que ocurren durante el control biológico de *R. necatrix*, y ayudar a diseñar futuros experimentos de transcriptómica, proteómica y/o metabolómica para profundizar en un futuro sobre el conocimiento a nivel molecular de las interacciones multitróficas que tienen lugar en un suelo supresivo enmendado con cáscara de almendra compostada.

CHAPTER I

GENERAL INTRODUCTION

1. Soil general features

Soil Microbial Ecology is the study of the microbial interactions in soil environments, including plants, animals and each other. Soil microbial communities (or soil microbiome) are large, diverse (>10^9 individual cells and >10^6 distinct taxa per gram of soil) and responsible for essential functions to plant growth and global environment. These soil microorganisms participate in the biogeochemical cycling of nutrients and organic matter, and can improve plant performance and soil quality, key issues for agroecosystem self-sustainability (Bulluck *et al.*, 2002).

Soil quality could be defined as the capacity of the soil to function within ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant and animal health. Indicators of soil quality are commonly classified in physical, chemical and biological parameters, which depend directly on soil management practices and maintenance of adequate soil organic matter (Doran and Parkin, 1994). The maintenance of soil quality is essential for crops yield. Several crop management practices could help to promote the right balance of soil quality measures based on ecological principles. Different examples of these techniques could be crop rotation, cover crops incorporated as green manures, minimal tillage practices, soil solarization and/or applications of external organic inputs (Hadar and Papadopoulou, 2012). Among them, the addition of organic matter to soils can improve soil quality by affecting many parameters, such as soil aeration, structure, drainage, moisture, nutrient availability (Bulluck *et al.*, 2002), in summary, soil microbial ecology.

Organic soil amendments or mulches, including composted or uncomposted plant residues and animal manures, differently affect on the balance of soil microbiome and plant diseases. Some types of mulches could have a negative effect in soils, as reported in several studies, where the impact of animal manures incorporation of soils can sometimes increase the incidence and severity of plant diseases (Aryantha *et al.*, 2000). Nevertheless, numerous researches have been demonstrated the improvement of plant

health and crop yield, as well as, the suppressive effect against soilborne diseases of composted materials used as organic amendments (Masry *et al.*, 2002; Liu *et al.*, 2007; Kyselková *et al.*, 2009; Mendes *et al.*, 2011; Pane *et al.*, 2013; Bonilla *et al.*, 2015). Several studies defend that the addition of plant residues to soil, in general, improves soil structure and soil health, improving plant growth and decreasing the disease incidence of plant pathogens (Garbeva *et al.*, 2004; Bonilla *et al.*, 2012a). Particularly, application of organic mulches could increase soil bacteria biomass, who normally have overlapping physiologies that promote the complexity of the soil food web. Different soil bacteria are critical to the maintenance of soil function displaying an important role in structure formation, decomposition of organic matter, toxin removal and biogeochemical cycling (Doran and Parkin, 1996). In addition, some groups of bacteria could play key roles in suppressing soilborne plant diseases and in promoting plant growth (Weller *et al.*, 2002).

2. Suppressiveness-induced soils

Following the classical definition of Cook and Baker (1983), a suppressive soil provides an environment in which plant disease development is reduced, even when the pathogen is favored by the presence of a susceptible host. Soil suppressiveness against soilborne plant pathogens, induced by the application of organic composts, is a widespread and ubiquitous phenomenon. Nevertheless, a single compost did not show a significant disease suppression against all pathogens and that pathogens were not affected similarly by all composts (Hadar and Papadopoulou, 2012).

We can find several approaches with examples of suppressive soils to different pathogens. For example, soils naturally suppressive to *Thielaviopsis basicola*, causative agent of black root rot of tobacco, were described by Stutz *et al.*, (1986). In this case, disease suppressiveness is a proven property conferred by specific microbial agents that are favored by amendment, specifically, fluorescent Pseudomonads isolates

from these soils producers of antifungal compounds such as 2,4-diacetyl-phloroglucinol (DAPG) and/or hydrogen cyanide (HCN).

Additionally, different examples of suppressiveness have been described in literature. The effect of suppressive soil against *Rhizoctonia solani*, a fungal pathogen of many crops including sugar beet, potato and rice was described by Mendes *et al.* (2011). Results suggest that the complex phenomenon of disease suppressiveness of soils cannot simply be ascribed to a single bacterial taxon or group, but is most likely governed by microbial consortia, suggesting that a general suppressiveness could be induced by a large metabolically active microbial community. Similarly, Pane *et al.*, (2013), described the ability to control damping-off diseases caused by *Rhizoctonia solani* and *Sclerotinia minor* by different composted amendments. In this study, ecological relationships between organic carbon molecular distribution and microbial structure may contribute to discriminate a suppressive compost affecting to microbial community functions.

Another example of soils harbouring microorganisms that can efficiently suppress pathogens has been also described by Latz *et al.* (2016). Their results indicate that plant communities performed soil-disease suppression via changes in abiotic soil properties and the abundance of bacterial groups including *Actinobacteria*, *Bacillus* and *Pseudomonas*.

All these studies reflect that plant disease suppression could be considered frequently a direct result of the microbial activities (Hoitink *et al.*, 1986). For this reason, studies focusing on microbial ecology in soil suppressiveness generated by amendments of organic matter could contribute to the identification of the sources of variability and models of action of the microbial communities. However, a lack of ecological theories to guide research in microbial ecology in complex environments has limited the progress in this field of study over the years (Hadar and Papadopoulou, 2012).

2.1. Microbial community from suppressive soils

The community structure of soil microbiome is influenced by many soil variables, among others, location, structure, particle size, mineral composition, temperature, humidity and agricultural practices (Bailey and Lazarovits, 2003). Moreover, depends on plant species and cultivar, developmental stage, plant growth substrate and stress factors. The composition of root exudates could vary, affecting directly to microbial biomass and activity around the roots. For this reason, plants are able actively selecting for their bacterial rhizosphere (Berg and Smalla, 2009; Berg *et al.*, 2014). Nevertheless, bulk soil microbial composition is essential to rhizosphere community because directly influences the physical-chemical parameters of root zone and the available range of microorganisms which interact with the plant and each other (Bonilla *et al.*, 2012a).

In a soil community we can find different groups of microorganisms which may be beneficial or deleterious depending on its abundance such as putative human and plant pathogens and plant beneficial organisms (Mendes *et al.*, 2013). Into the beneficial group of microbes we could found bacterial strains with beneficial effects on plant commonly known as plant growth-promoting rhizobacteria (PGPR) and biocontrol strains, who could use several mechanisms to suppress or reduce the severity of some plant diseases. The four most studied mechanisms used by soil microorganisms in order to induce plant protection by growth-promotion or disease control, are successful competition for C and nutrients, production of antimicrobial compounds toxic against pathogen, predation/parasitism by production of lytic enzymes and induction of plant resistance to disease (summarized in Figure 1).

2.1.1. Competition

Disease suppression based on competition could be related to microbial metabolic activities and it is controlled by the availability and rate of utilization of nutrients and

energy sources (Hadar and Papadopoulou, 2012). Several examples could be displayed. Mandelbaum and Hadar (1990) studied that competition for C source was suggested as a mechanism of suppression of *Pythium aphanidermatum* due to oospores could not germinate because of competition by microbial community. In this case, repetitive inputs of glucose and asparragine desequilibrated the microbial population and reduced the suppression phenomenon. Some studies showed *Fusarium oxysporum* was highly susceptible to competition for nutrients because application of organic matter favoring the increase of competitive microorganisms that could have an antagonistic activity against the pathogen (Alabouvette *et al.*, 2006). In other cases, competition for other type of nutrients such as iron can occur. For example, competition for iron can suppress *Fusarium* wilt in radish (De Boer *et al.*, 2003) because iron is important due to its extremely low solubility, making it often a limiting element in soil and rhizosphere. For this reason, some microorganism secrete siderophores that chelate iron harming the pathogens growth.

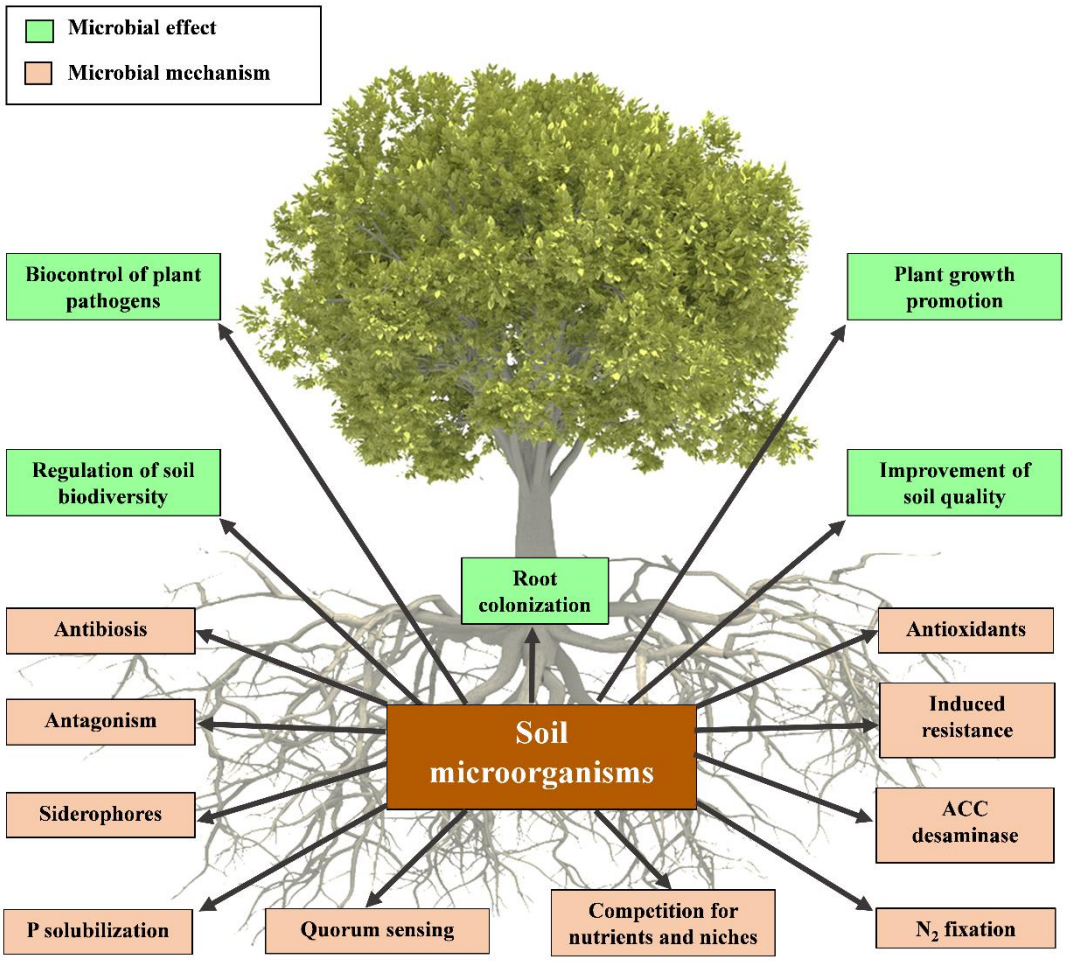


Figure 1: Microbiome functions in the suppressive soil-plant system. Effects of bacterial mechanisms used to promote plant growth, control plant pathogens and maintenance of soil quality. P, phosphorus; N_2, atmospheric nitrogen; ACC, 1-aminocyclopropane-1-carboxylate deaminase.

2.1.2. Antimicrobial compounds

The production of secondary metabolites such as antibiotics, biosurfactans or volatiles compounds have an antagonistic effect against pathogen. Antibiotics are low-molecular weight compounds produced by microorganisms that are deleterious to the metabolism or growth of other microorganisms. For example, pyrrolnitrin (PRN) is a secondary metabolite produced by *Pseudomonas* and *Burkholderia* spp. strains with strong antifungal activity to control plant diseases caused by fungal pathogens (Hammer *et al.*, 1997); 2,4-diacetylphloroglucinol (DAPG) is a secondary metabolite implicated in the primary mechanism of suppression of takeall of wheat by *Pseudomonas fluorescens* strain CHA0 (Weller *et al.*, 2007). *Bacillus* spp. are able to produce more than two dozens of antibiotics with antimicrobial effects (Pérez-García *et al.*, 2011). These antimicrobial compounds are peptides that are either ribosomally synthesized and post-translationally modified (lantibiotics and lantibiotic-like peptides) or non-ribosomally generated. All of these compounds have different actions: non-ribosomally produced lipopeptides are involved in biofilm and swarming development whereas lantibiotics function as pheromones in *quorum sensing* (QS; Stein 2005).

In the other hand, biosurfactans are amphiphilic compounds that can damage cellular membranes, thereby causing leakage and cytolysis. A wide range of structurally different biosurfactants have been identified to date, including glycolipids, lipopeptides, polysaccharides, proteins and lipoproteins, or mixtures, implicated in interactions with coexisting organisms, including bacteria, fungi, oomycetes, protozoa, nematodes and plants (Raaijmakers *et al.*, 2010).

Furthermore, volatile compounds, with an inorganic or an organic (VOCs) chemistry, are commonly produced by bacteria as communication molecules (Effmert *et al.*, 2012), but some of them have been described by its suppressive effects. For example, hydrogen cyanide (HCN) was an inorganic volatile compound produced by several

Pseudomonas spp. strains with antagonistic activity against different pathogens first described by Castric *et al.* (1975). Different studies have described the key role of volatile organic compound in antagonistic events, as described Zhou *et al.*, (2007) who isolated soil bacteria from different groups with VOCs production involves in soil fungistasis. The VOCs emitted from different bacterial antagonists (*Pseudomonas* spp., *Serratia* spp., *Stenotrophomonas* spp. and *Bacillus* spp.) negatively influence the mycelial growth of the soilborne phytopathogenic fungus *Rhizoctonia solani* (Kai *et al.*, 2007). Moreover, the antifungal volatiles produced by *Collimonas* spp. could play an important role in performing its mycophagous lifestyle (Garbeva *et al.*, 2014).

2.1.3. Lytic enzymes

Production of lytic enzymes allows microorganism to degrade several components that are present in the cell walls of fungi and oomycetes. Wide variety of bacterial lytic enzymes are known, including cellulases, glucanases, proteases and chitinases. The parasitic activity of various microorganisms toward plant pathogens involves recognition of the pathogen by the antagonist and excretion of several cell-wall-degrading enzymes to penetrate the hyphae of its pathogen (Hadar and Papadopoulou, 2012). This type of antagonism cause the death of the plant pathogen resulting in reduction of its inoculum density. Previous work based on isolated microbial community members from chitin-amended soils, showed the increase in bacterial isolates from *Streptomyces* spp., *Stenotrophomonas* spp. and *Bacillus* spp. strains and revealed the molecular diversity of the chitinases present in this soil (Cretoiu *et al.*, 2013).

2.1.4. Induced plant health

Different mechanisms are involved in promoting plant growth, such as, increase of tolerance to abiotic stresses, fixation of atmospheric nitrogen to ammonia, secretion of several plant hormones (auxins, cytokinins and gibberellins), production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, production of volatiles products, induction of local and systemic resistance (ISR), etc. Most of them are involved in the stimulation of the suppressiveness effect of the soil. Most of these processes are performed by the combined activities of microorganisms with different functional roles. In this sense, many different rhizospheric bacteria in a variety of plant species, could induce systemic resistance (ISR) (Bakker *et al.*, 2013). For example, the use of ISR-promoting PGPR *Bacillus* and *Pseudomonas* spp. strains could be a feasible strategy for the integrated control of cucurbit powdery mildew caused by *Podosphaera xanthii* (García-Gutiérrez *et al.*, 2012).

In soils, all of these mechanisms could take place at same time in the same niche due to continuous interaction among each other. One of the most recognized interaction mechanism are those under *quorum sensing* and *quorum quenching* (QQ) regulation. Microorganisms use QS to sense the existence of neighboring species and coordinate their behaviors, such as virulence, competence, symbiotic interactions, motility and biofilm formation, using different signal molecules, including, among others, N-acyl homoserine lactones (AHLs) and quinolones in Gram-negative bacteria or oligopeptides in Gram-positive strains (Song *et al.*, 2014). In other hand, diferent types of enzymes and chemicals can act as QS inhibitors, causing *quorum quenching*, the disruption of QS signaling (Grandclément *et al.*, 2016).

3. Single strain vs. community approaches

Along years, cultured bacterial isolation from suppressive soils have been performed in order to get insight on the mode of action finally responsible of soil suppressiveness. Several approaches studied the implication of single strains in biocontrol and/or promotion of plant growth. However, soil microorganisms do not act as individuals but rather act as a dynamically changing microbial community, where all cells interact and communicate with one another (De Roy *et al.*, 2014). Understanding the microbial ecology is essential in order to advance the possible biotechnological application of soil microorganisms but extreme complexity of natural microbiomes difficulted this knowledge. A promising way to overcome the difficulties associated with studying natural communities is to create artificial consortia that retain some of the key features of their natural counterparts. These can then act as a model system to assess the role of key ecological, structural and functional features of communities in a controlled way (Großkopf and Soyer, 2014).

Teague and Weiss (2015) reported that different members of a consortium could assume different responsibilities, increasing overall productivity and allowing more complex behavior than with single strains of a monoculture. In this way, the construction of artificial microbial consortia opens a new horizon in synthetic biology, research field that employs engineering principles to program novel biological systems (Song *et al.*, 2014).

Nevertheless, previous to the design of a synthetic consortia, different analysis should be performed. The partner members into a microbial community employed a diverse set of molecular interaction mechanisms to synchronize their behaviors. The use of different approaches to understand these interactions is an important key to advance in Microbial Ecology knowledge (Figure 2).

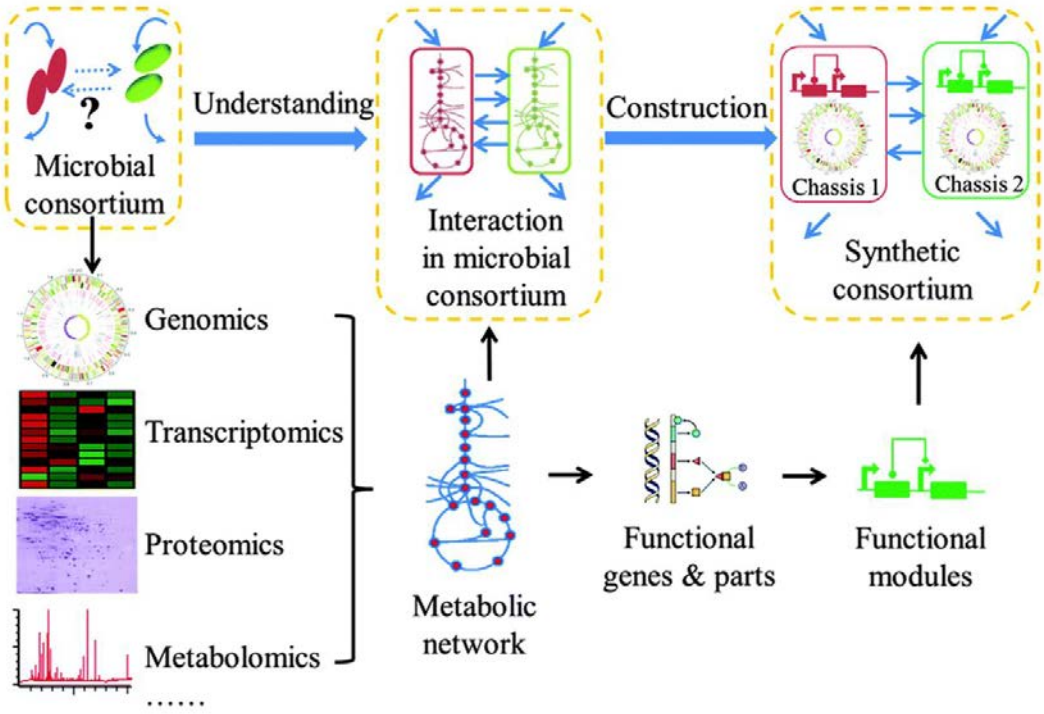


Figure 2: Strategies for systematic analysis and rational design of synthetic microbial consortia. The molecular mechanisms of interactions between microorganisms in microbial consortia could be elucidated using systems biology approaches (genomics, transcriptomics, proteomics, metabolomics, etc), which could provide insights into the design and construction of functional genes, parts, modules and the entire synthetic consortia. **Adapted from Song *et al.*, 2014. *Chem. Sci. Rev.***

Different type of interactions between microorganisms could occur in the consortia. Microbial communication may occur by contact-based interactions by which the exchange of biomolecules can occur via physical cell-cell contact and by contact-independent interaction when the exchanges of metabolites and information signals between microorganisms can take place. For example, Ghazali *et al.*, (2004) described the ability of *Bacillus* and *Pseudomonas* spp. consortium to remove hydrocarbons in contaminated soils when they were mixed in equal proportions and inoculated. In other study, a cellulose-degrading mixed culture consisting of five bacterial strains was defined (Kato *et al.*, 2005). In this case, various types of contact dependent relationships, positive and negative, were considered essential for the stable

coexistence of the members of the mix. In other hand, previous studies showed that contact-independent interaction may occur using *quorum sensing* molecules or a wide range of small molecules which allow the establishment of cell-cell interactions. The QS system, in particular the AHL-bases cell-cell communication, have been widely adopted in the construction of many synthetic microbial ecosystems in order to control population density and synchronization of process (Song *et al.*, 2014). In the other hand, the use of small molecules as cell-to-cell communication molecules have also been described, for example the syntrophic association that take place between *Desulfovibrio vulgaris* (Bacteria) and *Methanococcus maripaludis* (Archaea) in co-culture, where *D. vulgaris* produce organic carbon sources as electron carriers which *M. maripaludis* use as electron donor (Stoylar *et al.*, 2007) or the production of phenazine by members of fluorescent Pseudomonads that control their own expression of specific genes (Dietrich *et al.*,2006).

3.1. Work with microbial communities

Understanding soil microbiology is necessary to relate plant-pathogen suppression and microbiome dynamics. However, investigation of microbiome dynamics, rather than single microbial species, severely limits the applicability of classical ecological models, due to the complexity of the natural communities.

Historically, cultivation-based techniques to assess diversity in soil environment rely mainly on the dilution-plating technique (coupled with the use of selective media) and biochemistry and morphological characterization (Jeewon and Hyde, 2007). Cultural methods, coupled with biochemistry tests are the earliest techniques used to detect exactly which taxon is present (identification) in soil samples. But generally, it appears that both methods have specific bias, as data generated is largely dependent upon the methodologies involved and normally, have a number of limitation which difficult a proper diversity assessment. These methods have commonly been used because of

their simplicity and low cost but they tend to overestimate culturable species. Most of these methods result in isolation of only the most common and abundant microorganisms, those can utilize the energy source under the physical and chemical limitations of the growth medium. It is thought that only a small fraction (0.1 to 10%) of microorganism existing in the nature can be cultured artificially. Moreover, during these processes, the risk of culture contamination is always high and in most cases the fast-growing organism will overgrow others and occupy the whole medium (Jeewon and Hyde, 2007).

For these reasons, molecular tools had evolved to offer an opportunity to monitoring totally microbial populations (Figure 3).In the past decades, one of the most striking events in the field of Microbial Ecology has been the development of -omics sciences: Metagenomics, Metatranscriptomics, Metaproteomics and Metabolomics.

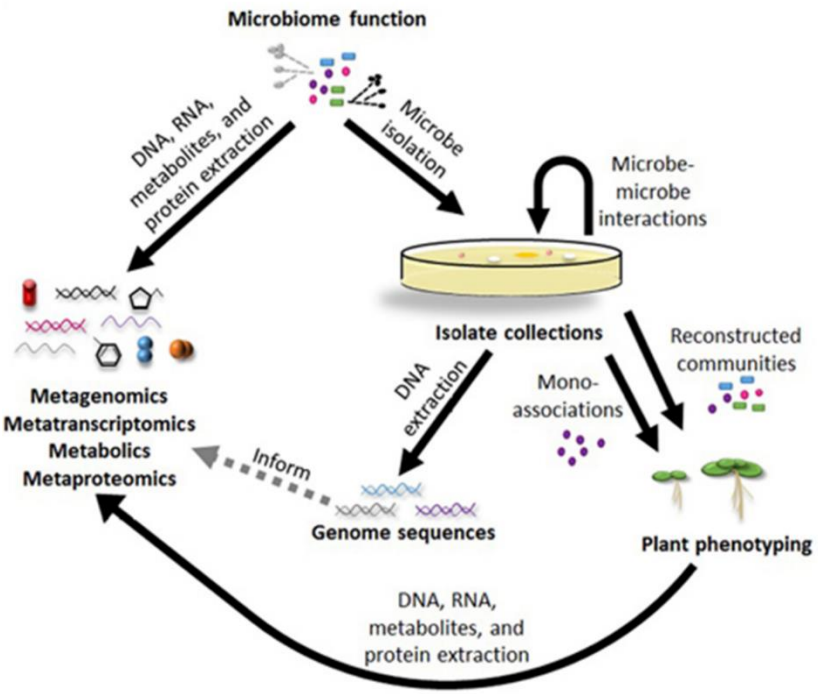


Figure 3: Methods for characterization of plant microbiome function. Proposed pipelines to integrate the data from culture-independent and culture-dependent methods together to address functions of plant microbiomes and the individual types of microbes found within them. **Adapted from Lebeis, 2014. Front. Plant Sci.**

Metagenomics is defined as the direct analysis of genomes contained in an environmental sample (Thomas *et al.,* 2012). This technique provides access to the functional gene composition of microbial communities and phylogenetic diversity based on 16S rRNA gene and ITS (internal transcribed spacer) regions, given the potential linkages between genomic function and phylogeny for uncultured organism, and evolutionary profiles of community function and structure. Profiling of the microbial community could be essential to get insight about who and how a soil community are formed. Traditionally, in order to identify which groups of microorganism are represented in a community, methods that not rely on sequencing were used, such as electrophoretic separation using "denaturing gradient gel electrophoresis" (DGGE; Muyzer *et al.,* 1993), amplification with specific fluorescently labeled primers digested with restriction enzymes to reveal "terminal restriction fragment polymorphism" (TRFLP; Liu *et al.*, 1997) and/or "automated ribosomal intergenic spacer analysis" (ARISA), where the lengths of the variable spacer regions separating the ribosomal genes are compared (Danovaro *et al.,* 2006).

Nowadays, metagenomics approaches allow the sequencing of 16S rRNA genes and ITS regions in order to assess the diversity of microbial communities. DNA sequences are amplified using universal primers of conserved sequences that flank most variable regions. Each group of PCR amplicons that shares a similar or identical variable region is considered an "operational taxonomic unit" (OTU) and assumed to be equivalent to a bacterial species or genus (depending on the degree of sequence similarity). Various methods can be used to resolve or identify the OTUs, based in a pipeline of tools to facilitate the identification and calculation of some parameters such as relative abundance, species richness or evenness (Hirsch *et al.* 2013) (Figure 3).

A few years ago, the method to assess amplicon diversity involved cloning and sequencing procedures. Limitation to the numbers that can be processed leaded that only the predominant members of the community were identified. In this sense, the

use of high throughput next generation sequencing (NGS) methods (e.g.: Ilumina MiSeq / HiSeq, Ion Torrent PGM, Roche 454 FLX Junior, PacBio RS II) to directly sequence PCR amplicons, facilitate the knowledge of the microbial community faster (and cheaper) that previous methods involves in cloning and sequencing.

This technology has also been applied to functional microbial community analysis, using functional gene arrays (FGAs). In this case, NGS technologies allow capturing sequences for targeted genes with available primers. For example, GeoChip® is a comprehensive functional gene array targeting hundreds to thousands genes of different families that play important roles in various biogeochemichal processes, enabling researchers to comprehensively analyze the functional diversity, composition and structure of microbial communities in various environments (Tu *et al.*, 2014). GeoChip® was developed for broader applications in analyzing biogeochemical processes and microbial responses to environmental perturbations with gene families involved in carbon, nitrogen, sulphur and phosphorous cycles, organic remediation, metal reduction and antibiotics, environmental stress responses, bacteriophages and virulence processes (http://www.glomics.com/gch-tech.html). Metagenomics approaches can be complemented with metatranscriptomic, metaproteomic and metabolomic approaches.

Metatranscriptomic facilitates insight into the potential expression of genes at the time of the sampling (Carvalhais *et al.*, 2012), providing a real transcriptional profile that correspond to discrete populations within a microbial community. This information can indicate the potential activities of complex microbial community and mechanisms that regulate those, at the time of sampling. But there are some key limitations inherent to metatranscriptomics, such as the half-lives of mRNA molecules, additional amplification steps of starting material which can skew the amplification towards most repeated sequences, and the assigned identification sequence step with comparison

with publically available databases instead of databases generated using metagenomics data from the same or highly similar communities (Carvalhais *et al.*, 2012).

In the other hand, metaproteomic approaches allow the study of the proteins recovered directly from environmental samples at a given point of time (Wang *et al.*, 2011). These type of approaches complemented the metatranscriptomic studies, favouring the identification of proteins present in soil samples, and gaining insight in the role of them in soil ecological processes (Wang *et al.*, 2011) (Figure 3). In this sense, recent studies suggest that knowledge of volatile compounds produced by microorganisms could help us to get insight in this purpose. Concretely, volatile organic compounds (VOCs) are compounds present in soils, characterized by low molecular weight and high vapor pressure that can act as mediator molecules of inter- and intraspecies relationships (Effmert *et al.*, 2012). Bacterial VOCs can be considered as important components of the complex interactive mechanisms among bacteria and between bacteria and other organism, including eukaryotes, in their natural environments (Popova *et al.*, 2014).

In order to obtain a complete analysis, metabolomics experiment could be performed, capturing the complexity of metabolic networks via the comprehensive characterization of the small-molecule metabolites (such as aminoacids, sugars, and lipids) in biological systems and how they vary in response to a variety of stimuli. Some researches, as Jones *et al.*, (2014), defend that metabolomics has a large practical advantage over other "omic" systems due to a fully annotated genome is not required for analysis, and analytical methods are transferable between species. They proposed the term "community metabolomics" for the application of metabolomics technique to the study of the entire community of a soil sample, obtaining the metabolic profiling that can be used to assess the changes in biochemical profiles of soil communities (Figure 3).

4. Case of study: the avocado crop

Avocado is an appreciate fruit around the world, due to its excellent nutritional value (https://www.avocadocentral.com). The main avocado-producing and exporting areas are located in South America, but in Europe, we could find avocado crops in the Mediterranean region, mainly restricted to the south of Spain and Portugal. In fact, Spain is the main European country producing avocado, with a production of 69400 tons in 2013 with around a 94% of the total Spanish production exported to the European Union (FAOSTAT, http://www.fao.org/faostat/en/#data).

In the Iberian Peninsula, the avocado crop is mainly located in the Andalusian coast (Málaga and Granada) where the area of planting has reached 10000 Ha. In this zone, the production during 2014-2015 has been 47500 tons (40000 tons in 2013-2014), selling to national trade around a 19% and exporting to EU a 78% (mainly France, United Kingdom and Germany; ASAJA Málaga, http://www.asajamalaga.com/?n=1596).

4.1. Avocado root rot diseases

Soilborne disease caused by phytopathogenic organisms are one of the main problems for avocado tree (*Persea americana*, Miller). In southern Spain, the most destructive avocado root rot diseases are caused by *Rosellinia necatrix* and the oomycete *Phytophthora cinnamomi*. *Phytophthora* root rot is the main disease affecting avocados across all continents around the world causing severe losses in fruit production (Pérez-Jiménez, 2008). White root rot caused by *R. necatrix* is the main root disease in the Mediterranean region, with occur favorable environmental conditions that has turned this disease into one of the main limiting factors for avocado production. Moreover the currently areas used for avocado production were previously occupied by other susceptible plant hosts such as vineyards and almond crops (Pérez-Jiménez, 2008).

Avocado trees affected by *R. necatrix* show aerial symptoms as wilting and drying of leaves and a decline in the general vigour of the tree. The evolution of aerial symptoms occur very quickly and death of an adult tree can occur in a few weeks after the appearance of the first foliar symptoms (Pérez-Jiménez, 2008).

The integrated control of the disease have been studied since 90's decade. First, control of white root rot caused by *R. necatrix* in other susceptible host as apple, tea or vineyards, was mainly conducted by the use of antifungal compounds. For this reason, fungicides benomyl, carbendazym, methyl thiophanate and fluazinam have been tested in avocado crops. Fluazinam is the most promising fungicide tested *in vitro* and *in vivo* (López-Herrera and Zea-Bonilla, 2007). However, *R. necatrix* have shown to be resistant to several fungicides. Additionally, there are not available tolerant rootstocks to this soilborne pathogen.

As alternative to use of chemical compounds, the physical treatment based in soil solarization was implemented successfully in avocado crops in order to eliminate the high temperature sensitive inocula of *R. necatrix* in the top layers of soil (López-Herrera *et al.*, 1998). Moreover, biological control approaches based on the application of *Trichoderma* and/or bacterial strains have been also studied (Ruano-Rosa *et al.*, 2014) but remain experimental.

Bacterial isolates from the rhizosphere of healthy avocado trees were obtained (Cazorla *et al.*, 2006, 2007; Pliego *et al.*, 2007, 2008), and tested for antagonistic activity against *R. necatrix* due to antagonism is a prevalent trait in the biocontrol bacteria selected by a direct protection strategy of avocado plants (Gónzalez-Sánchez *et al.*, 2010). Several strains belonging to the genera *Bacillus* and *Pseudomonas* have been selected. For example, *Pseudomonas chlororaphis* PCL1606 is an antagonistic rhizobacterium that produces lipases, proteases, siderophores and the antifungal metabolites HPR (2-hexyl 5-propyl resorcinol), PRN and HCN and can also control the avocado white root rot by competition for the same niche and presumably, also for

root exudate nutrients (Calderón *et al.*, 2014). *Pseudomonas chlororaphis* PCL1601 produced proteases, lipases, siderophores, HCN, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN), compounds with antagonistic activities (Cazorla *et al.*, 2006). A third strain, such as *Pseudomonas pseudoalcaligenes* AVO110, was isolated due to its efficiently colonization of avocado root tips (Pliego *et al.*, 2008), displaying plant protection by competition for nutrients and occupying the same niche that the pathogen. For this reason, many works have been focused in the study of *Pseudomonas* spp. strains producer of different antimicrobial compounds, lytic exoenzymes and colonization patterns with implications in the biological control of the pathogen.

Furthermore, studies to test the effectiveness of single and combined applications of *Trichoderma* and rhizobacterial strains to control white root rot were performed. Compatibility between the combined species was observed and these combinations significantly improved the control of *R. necatrix in vitro* experiments. The combinations of *T. atroviride* strains with the strains *P.chlororaphis* PCL1601 and PCL1606 and *P. pseudoalcaligenes* AVO110 showed the best biocontrol activity against avocado white root rot (Ruano-Rosa *et al.*, 2014).

Furthermore, other groups of bacteria have been isolated and identified as antagonistic strains against *R. necatrix*. *Bacillus subtilis* PCL1608 and PCL1612, isolated from healthy avocado rhizosphere, presented biological control activity against different soilborne phytophathogenic fungi, including *R. necatrix*. These bacterial isolates were able to produce antifungal compounds involved in their antagonistic activity such as glucanases or proteases and the antibiotic lipopeptides surfactin, fengycin and iturin (Cazorla *et al.*, 2007). Other example is *B. subtilis* CB115, isolated from avocado soil, and with an excellent biological control activity against *R. necatrix*, and antagonistic ability against different soilborne phytopathogenic fungi. This strain also produced

several exoenzymes and antibiotics, such as fengycin and surfactin, and was able to induce plant-growth promotion (González-Sánchez *et al.*, 2010).

4.2. Organic amendments in avocado crop

Nowadays, demand for organically produced food has increased and many consumers have expressed concern over pesticide residues. Food and environmental safety are often-cited reasons for the use of alternative soil management based on the prevention of the appeareance of the pathogen (Bulluck *et al.*, 2002). For this reason, it is important the usage of preventive management practices such as the use of organic amendments that changes the microbial and physicochemical characteristics of soils.

Historically, the use of organic amendments or mulches in avocado crops has produced beneficial effects such as increased root growth and health, reduced plant stress and increased avocado yield (Wolstenholme *et al.*, 1997; Moore-Gordon *et al.*, 1997; López *et al.*, 2014). These phenomena are directly related with the effect of the organic matter input in physicochemical and biotic properties related to microbial diversity, structure and activities (Bonilla *et al.*, 2012b).

The use of an appropriate mulch is crucial in organic management of most woody perennial crops such as avocado, because it provides several environmental and agronomical advantages. Moreover, and from a sustainable perspective, residual organic materials from nearby crops or agro-industrial activities would be the best option as sources of mulch. For this reason, farmers in the area have applied composted almond shells to avocado crops, maintaining the C balance due to its richness in lignin and phenolic compounds and influencing in soil microbial enzyme activity (Jafari et al, 2012). Composted almond shells are obtained as a residue and its ligneous character makes this waste suitable to obtain activated C or biomass fuel (Fernández *et al.*, 2012). A work on, the possible uses of the almond shells, made this waste highly recommended as mulch due to its long lasting, availability, low cost and resistance

(Esfahlan *et al.*, 2010). In our case of study, the avocado is a subtropical crop whose adaptation to the growth in non-subtropical climates is highly dependent to the presence of a decomposing litter layer, in which its feeder roots proliferate. In this sense, composted almond shells act as reinforcement to the natural dead leaf layer, which is left as a traditional agriculture practice (Wolstenholme *et al.*, 1997).

Previous studies by culture-dependent approaches analyzed the population size of several groups of microorganisms in conventional and organic orchards, which use different organic amendments. Bacterial community structure was studied by denaturing gradient gel electrophoresis (DGGE) showing that all of the amendments used affect the soil microbiome structure. The largest effects were shown by commercial composts, especially animal compost that enhance the population sizes of some microbial groups and affecting bacterial community structure in superficial and deep soil layers but stronger in the superficial layer of the avocado soil (Bonilla *et al.*, 2012a).

Mesocosms analysis performed with two-year-old avocado trees growing in soil treated with composted organic amendments and then used for inoculation assays, showed that all of the organic treatments reduced disease development in comparison to unamended control soil, especially the amendments of yard waste (YW) and composted almond shells (AS). The YW had a strong effect on microbial communities in bulk soil and produced larger population levels and diversity, higher hydrolytic activity and strong changes in the bacterial community composition of bulk soil, suggesting a mechanism of general suppression. Amendment with AS induced more subtle changes in bacterial community composition and specific enzymatic activities, with the strongest effects observed in the rhizosphere. Even if the effect was not strong, the changes caused by AS in bulk soil microbiota were related to the direct inhibition of *R. necatrix* by this amendment, most likely being connected to specific populations able to recolonize conducive soil after pasteurization (Bonilla *et al.*, 2015) (Figure 4).

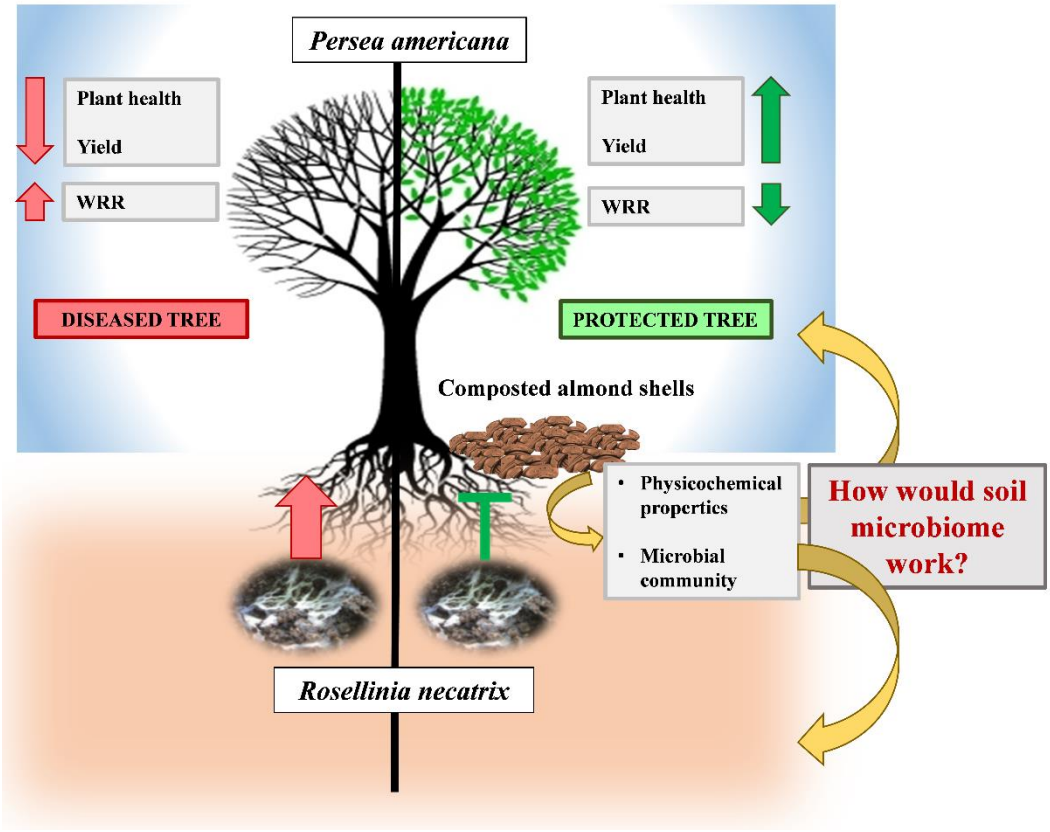


Figure 4: State of the art. Soil suppressiveness induced by the application of composted almond shells as organic amendment against *Rosellinia necatrix*, in avocado crops.
WRR, avocado white root rot.

OBJECTIVES

The application of composted almond shells induce soil suppressiveness against avocado white root rot, caused by the phytopathogenic fungus *Rosellinia necatrix*. In this work, we aim to analyze the composition and function of the microbial community in this suppressive-induced soil. Furthermore, the isolation and characterization of relevant group of microorganisms from this soil, could allow the selection of new biological control agents against this soilborne pathogen. Additionally, we will use bacterial strains previously described by their biological control activity against this fungus, in order to improve the knowledge of the community interactions that take place during biological control of this disease.

1.- Unravelling the avocado field soil microbiome composition, and its role in the soil suppressiveness against *Rosellinia necatrix*.

2.- Isolation and characterization of new biological control agents against *Rosellinia necatrix,* belonging to a relevant bacterial group from a suppressive-induced soil revealed in previous objective.

3.- Use an artificial bacterial consortium of selected biological control strains, in order to understand the multitrophic interactions occurring in avocado roots.

OBJETIVOS

La aplicación de cáscara de almendra compostada induce supresividad frente a la podredumbre blanca radicular del aguacate, causada por el hongo fitopatógeno *Rosellinia necatrix*. En este trabajo, pretendemos analizar la composición y funcionalidad de la comunidad microbiana de estos suelos enmendados y su papel en la supresividad. El aislamiento y caracterización de ciertos grupos concretos de microorganismos desde este suelo supresivo, nos permitirá además seleccionar nuevos agentes de control biológico frente a dicho patógeno. Finalmente, utilizaremos cepas bacterianas con actividad de control biológico, para aplicarlas como un consorcio bacteriano artificial, e iniciar el estudio de las posibles interacciones que tienen lugar durante el control biológico de esta enfermedad. Y para ello planteamos los siguientes objetivos concretos:

1.- Caracterizar la composición del microbioma de un suelo de cultivo de aguacate, y su papel en la supresividad frente a *Rosellinia necatrix*.

2.- Aislar y caracterizar nuevos agentes de control biólogico frente a *Rosellinia necatrix* a partir de grupos bacterianos relevantes en la supresividad del suelo enmendado puestos de manifiesto en el objetivo anterior.

3.- Utilizar un consorcio bacteriano artificial de cepas con actividad de control biológico, para profundizar en el conocimiento de las interacciones multitróficas que tienen lugar en la raíz de aguacate.

CHAPTER II

Microbial profiling of a suppressiveness-induced agricultural soil amended with composted almond shells

PUBLISHED IN:

Vida, C., Bonilla, N., de Vicente, A. and Cazorla, F.M. (2016) Microbial profiling of a suppressiveness-induced agricultural soil amended with composted almond shells. *Front. Microbiol*. 7:4. doi:10.3389/fmicb.201600004

Abstract

This study focused on the microbial profile present in an agricultural soil that becomes suppressive after the application of composted almond shells (AS) as organic amendments. For this purpose, we analyzed the functions and composition of the complex communities present in an experimental orchard of 40-year-old avocado trees, many of them historically amended with composted almond shells. The role of microbes in the suppression of *Rosellinia necatrix*, the causative agent of avocado white root rot, was determined after heat-treatment and complementation experiments with different types of soil. Bacterial and fungal profiles obtained from natural soil samples based on the 16S rRNA gene and ITS sequencing revealed slight differences among the amended (AS) and unamended (CT) soils. When the soil was under the influence of composted almond shells as organic amendments, an increase in *Proteobacteria* and *Ascomycota* groups was observed, as well as a reduction in *Acidobacteria* and *Mortierellales*. Complementary to these findings, functional analysis by GeoChip® 4.6 confirmed these subtle differences, mainly present in the relative abundance of genes involved in the carbon cycle. Interestingly, a group of specific probes included in the "soil benefit" category was present only in AS-amended soils, corresponding to specific microorganisms previously described as potential biocontrol agents, such as *Pseudomonas* spp., *Burkholderia* spp., or *Actinobacteria*. Considering the results of both analyses, we determined that AS-amendments to the soil led to an increase in some orders of *Gammaproteobacteria*, *Betaproteobacteria*, and *Dothideomycetes*, as well as a reduction in the abundance of *Xylariales* fungi (where *R. necatrix* is allocated). The combination of microbial action and substrate properties of suppressiveness are discussed.

1. Introduction

The enhancement of soil suppressiveness using organic amendments has been widely described, especially for soilborne diseases (Lazarovits *et al.*, 2001; Bailey and Lazarovits 2003; van Elsas and Postma 2007; Bonilla *et al.*, 2012a; Pane *et al.*, 2013). However, this effect can be extremely variable depending on the pathosystem and the environmental conditions, and there are even some examples of the amendment application increasing disease incidence (Termorshuizen *et al.* 2006; Janvier *et al.*, 2007). The soils that become suppressive soils provide an environment in which plant disease development is reduced, even in the presence of a virulent pathogen and a susceptible host (Hadar and Papadopoulo, 2012). This phenomenon could be induced as a direct result of the activity of microorganism consortia that are naturally established on soil after application of the amendment (Weller *et al.*, 2002).

As such, understanding the diversity, composition, structure, function and interactions of microbial communities is crucial to gain insight into the basis for suppressiveness mediated by this organic amendment (Janvier *et al.*, 2007). Approaches for studying microbial communities in the soil are complex. Thus, employing genomic approaches to understand which changes occur in soil could be a good alternative strategy to decipher the profiling of soil microbiota (Garbeva *et al.*, 2004).

The use of genomic techniques rely on PCR amplification of the conserved and variable regions of the microbial genome, commonly 16S ribosomal RNA (rRNA) for bacteria and 18S rRNA or internal transcribed sequences (ITS) for fungi, allowing for direct sequencing of these PCR amplicons using different high-throughput next-generation sequencing methods. Each group of PCR amplicons that shares a similar or identical variable region is considered an “operational taxonomic unit” (OTU) and is assumed to be equivalent to a microbial species or genus. The analysis of OTUs

provide information about the phylogenetic diversity of the soil microbial community (van Elsas *et al.*, 2007, 2008; Hirsch *et al.*, 2013; Koyama *et al.*, 2014).

Moreover, complementary techniques have arisen, such as microarrays, which have considerable potential in environmental microbial ecology, providing novel insights into how environmental factors affect microbial communities in various habitats (Hazen *et al.*, 2010; He *et al.*, 2012; Bai *et al.*, 2013; Zhang *et al.*, 2013; Tu *et al.*, 2014). The GeoChip microarray is a comprehensive functional gene array (FGA) targeting hundreds to thousands of different gene families that play important roles in various biogeochemical processes, enabling researchers to comprehensively analyse the functional diversity, composition, and structure of microbial communities in various environments. It is a powerful FGA-based technology that can be used to survey the functional diversity, composition, structure, metabolic potential/activity, and dynamics of microbial communities, and then link them with ecosystem processes and functions (Xie *et al.*, 2011; Xue *et al.*, 2013; Cong *et al.*, 2015).

Our research interest is focused on the avocado (*Persea americana* Mill.), for which southern Spain is one of the most relevant zones in the Mediterranean area for this crop. In this part of the world, one of the most limiting soilborne diseases affecting avocado trees is white root rot, caused by the fungus *R. necatrix* Prill. White root rot is considered to be an emergent threat to many woody crop plants worldwide (Pliego *et al.*, 2009, 2012).

The role of soil microorganisms in the plant protection have been broadly reported. Thus, different microbes can contribute to the biocontrol of avocado white root rot using different weaponry such as antagonism (*Pseudomonas chlororaphis* PCL1606 or Bacillus *subtilis* PCL1608; Cazorla *et al.*, 2006, 2007), competition for niches and nutrients (Calderón *et al.*, 2014), or induction of systemic resistance or predation (*Trichoderma* spp.; RuanoRosa and López-Herrera, 2009). These microorganisms can act as single or combined with other biocontrol agents against *R. necatrix* (Ruano-Rosa

et al., 2014). Other studies have reported the positive effect of the application of arbuscular mycorrhizal fungi to soil and the biocontrol activity on avocado (Hass and Menge, 1990; González-Cortés *et al.*, 2012).

During the past decades, several approaches have been implemented to achieve an integrated management of *R.necatrix*, including physical, chemical and biological control approaches (López-Herrera *et al.*, 1998; López-Herrera and Zea-Bonilla, 2007; González-Sánchez *et al.*, 2013). All of these approaches seem to be effective at the experimental level, and some of them have been proven to be effective under certain conditions. However, at the same time, traditional strategies of land management have improved, and some of these strategies could be considered useful approaches to fight against diseases in avocado management, thus increasing the weaponry available against white root rot (Bonilla *et al.*, 2012b).

One of these approaches is the use of organic amendments or mulches, which have produced beneficial effects for plants, including increasing health and yields in avocado crops (Moore-Gordon *et al.*, 1997; Wolstenholme *et al.*, 1997; Hermoso *et al.*, 2011). It has been previously shown that the application of such organic matter to avocado agricultural soil can affect soil physicochemical properties and microbial communities (Bonilla *et al.*, 2012a; López *et al.*, 2014). Additionally, organic amendments could play a critical role in global biochemical cycles (Bonanomi *et al.*, 2014) and could cause different effects, such as the improvement of soil fertility and the enhancement of natural suppressiveness of the soil against several phytopathogens (Cretoiu *et al.*, 2013). Several organic amendments have shown an obvious suppressive effect against another important avocado soil-borne phytopathogen, *Phytophthora cinnamomi* (Bender *et al.*, 1992; Downer *et al.*, 2001).

In a previous study, it was shown that different organic matter applied as a mulch to the avocado crop exhibited suppressive effects against white root rot (Bonilla *et al.*, 2015). Composted almond shells were one type of organic matter tested. The

application of composted almond shells as a mulch led to an enhancement of the bacterial composition and activities of the soil communities in relation to the observed suppressiveness (Bonilla *et al.*, 2015). The objective of the present study was to gain insight into the microbial profiling present in the amended soils showing suppressive ability against the avocado soil-borne phytopathogen *R. necatrix*. The use of different microbial approaches should uncover the microbial communities potentially involved in the suppressive phenotype.

2. Materials and methods

2.1 Field of study

Soil samples were obtained from an avocado crop field (cv. *Hass* avocado trees grafted onto cv. *Topa-Topa* seedling rootstocks) located at the Experimental Station 'La Mayora' (IHSM-UMA-CSIC, Málaga, Spain) on the coast of the Malaga Province (SE Spain). This experimental field of 2.5 km^2 (36°75'N, 4°04'O) contains 195 40-year-old avocado trees planted at 8 x 8 m. Selected avocado trees were grouped in pairs to facilitate their management. Sixteen pairs of trees were under ecological management (massive application of composted almond shells in 2002, 2007 and 2012), and another 16 pairs of trees were under conventional management (addition of mineral nutrients twice per year, as well as the application of herbicides and pesticides when necessary, López *et al.*, 2014) and without any organic amendment.

2.2 Soil sampling

Natural field soil samples allocated underneath of avocado trees unamended (CT) or amended with composted almond shells (AS) were taken to perform the different experiments. Soil samples were collected in April 2013, November 2013 and April 2014. Composite soil samples were taken from four different groups of paired trees with (AS) or without (CT) organic amendment and were randomly selected from throughout the avocado orchard. To obtain a composite soil sample, two sampling distal points at 1.5m around the trunk base for each tree of a pair of trees under the same treatment were selected; the upper layer of compost was carefully removed, and 5–10kg of soil samples (15cm depth) were collected per pair of trees and merged. Samples were placed in cold storage and transported to the laboratory. Samples of each type of soil were sieved through a 20mm mesh and immediately used for physicochemical and suppressivenessexperiments.ToperformDNAextractions,three soil samples (1 g each) from composite soil samples per each pair of trees were sieved again (2mm diameter) and processed independently. The remaining unused soil samples were stored at −80◦C.

2.3 Physicochemical analysis of soil samples

Physicochemical analysis of both types of soil samples were performed at Laboratorio Caisur S.L. (Granada, Spain) using standardized methodologies. Four subsamples from each field soil sample (AS and CT) were analysed independently. Soil tests included: soil texture, pH, conductivity, total cation exchange capacity (CEC), organic matter, organic C, nitric and total N, C/N, and macro- and micronutrients, including phosphorus, potassium, iron, manganese, copper and zinc (Table 1).

Table 1: Physicochemical analysis of natural avocado field soils used in this study. Characteristics of amended with composted almond shells (AS) and unamended (CT) agricultural avocado field soils

Parameter (unit)	AS	CT
•Soil texture	Sandy loam	Loamy sand
•pH (20°C)	7.20	7.55
•Conductivity (ms/cm)	1.15	2.0
•CEC (meq/100gr)	61.87	62.01
Ca^{2+}/Na^{+} ratio	84.46	56.35
•Organic matter (%)	30.66	25.60
•Organic C (%)	13.73	11.46
•Total N (%)	0.07	1.10
•Nitric N (ppm)	184	256
•C/N ratio	196.14	10.42
•Phosphorus (ppm)	33	25
•Potassium (ppm)	1120	700
•Iron (ppm)	44.0	6.4
•Manganese (ppm)	44.0	10.0
•Copper (ppm)	3.6	2.4
•Zinc (ppm)	38	34

2.4 Soil processing

To test the potential role of soil microorganisms in suppressiveness, we prepared three types of processed soils using different treatments: Field soils (raw soils), heat-treated soils, and complemented soils (Table 2). We applied a moist heat treatment to the field soil samples as previously described (Weller *et al.*, 2002), with slight modifications. Briefly, the heat treatment consisted of heating the soil in high moisture conditions at 100◦C for 20min in an autoclave. The soil was allowed to recover at 4◦C overnight. Then, we performed a second treatment step, heating the soil at 100◦C for 10min in high moisture conditions. After allowing it to cool, the soil was ready to be used

(Figure 5). Complemented soils were prepared with the purpose of observing the partial recovery of the microbial characteristics of the natural soil (Weller *et al.*, 2002). The complemented soil consisted of heat-treated soil mixed with natural raw field soil in a 9:1 (w/w) ratio (Table 2).

To evaluate changes in the culturable microbiota fraction during different times of the soil sample processing, counts of cultivable colony forming units (cfu) of bacteria and fungi per gram of soil were performed. For this, 2 g samples of soil obtained at the different key times during the process were suspended in 20 ml of sterile saline solution (0.85% NaCl) with 0.5 g of sterile gravel and mixed at 150 rpm for 30 min on an orbital shaker at room temperature. Ten-fold serial dilutions of the obtained suspensions were plated on Luria Bertani (LB) agar with 100 mg of cycloheximide per litre, to analyse the heterotrophic bacteria group, and on potato dextrose agar (PDA) with 50 mg of chlortetracycline and 1 ml of tergitol NP-10 (Sigma) per litre (Bonilla *et al.*, 2012a).

2.5 Suppressiveness assays

Suppressiveness assays against white root rot caused by the virulent strain *Rosellinia necatrix* CH53 (López-Herrera and Zea-Bonilla, 2007) were conducted using two different susceptible pathosystems, avocado (Cazorla *et al.*, 2006) and wheat (*Triticum aestivum*). The *R. necatrix* inoculum was produced on wheat seeds (Freeman *et al.* 1986). The seeds were soaked for 12 h in 250-ml Erlenmeyer flasks filled with distilled water. The flasks were autoclaved after excess water had been drained off. After sterilization, fungal disks of a 1-week-old culture of *R. necatrix* grown on PDA were placed aseptically in each flask. Flasks were incubated at 25°C for 2-3 weeks and were shaken every 2 to 3 days to avoid clustering of the seeds.

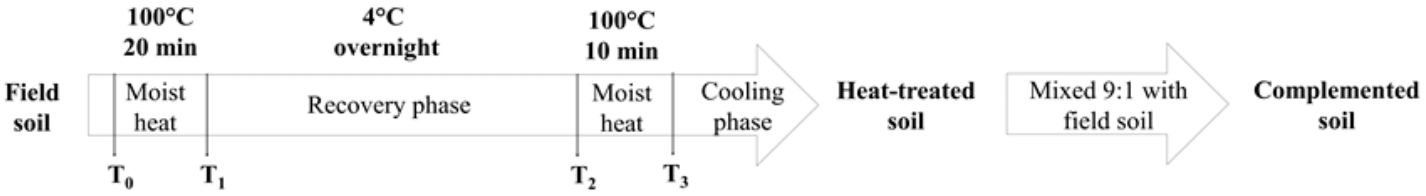


Figure 5: Processing scheme of the soil heat-treatment and complementation used in this study for the agricultural field soil samples. The same procedure was followed for both unamended soil and soil amended with composted almond shells. $T_{0\text{-}3}$ indicates sampling points to perform bacterial and fungal plate counts.

Table 2: Types of processed agricultural soils used in this study. A scheme of the processing is described in Figure 5.

Soil source	Treatment code	Details of processed soils
Amended with composted almond shells	AS	Natural field soil amended with composted almond shells mulching
	ASt	AS heat-treated soil
	ASc	ASt complemented with AS in 9:1 (w/w) ratio
	ASt+CT	ASt complemented with CT in 9:1 (w/w) ratio
Unamended and under conventional management	CT	Natural field soil unamended and under conventional management
	CTt	CT heat-treated soil
	CTc	CTt complemented with CT in 9:1 (w/w) ratio
	CTt+AS	CTt complemented with AS in 9:1 (w/w) ratio

Avocado/*R. necatrix test system*

Six-month-old commercial avocado plants were obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain). The roots from the avocado plants were disinfected by immersion in 0.1% NaOCl for 20 min and then washed twice (20 min) with sterile distilled water. Then, avocado plants were placed into square plastic pots (10.5 x 10.5 x 10.5 cm) containing 0.64 L of the sieved CT and AS types of soils. Fungal infection with *R. necatrix* was performed using wheat grains (4 holes of 2 cm depth were made per pot, 3 infected wheat grains were placed per hole) as previously described (Freeman *et al.*, 1986). Non-infected plants were used as controls. Three sets of fifteen avocado plants were tested per type of soil. The plants were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms of avocado white root rot were recorded on a scale of 0 to 3, and a disease index (DI) was calculated after 5 weeks using the previously described formula (Cazorla *et al.*, 2006).

Wheat/*R. necatrix test system*

Wheat seeds were disinfected by immersion in 0.05% NaOCl for 10 min, washed and then placed in darkness between pieces of moist filter paper in a growth chamber for 2-3 days at 25°C to induce germination. Then, germinated seedlings were disinfected again by immersion in 0.1% NaOCl for 20 min and washed (20 min) with sterile distilled water. Seedlings were placed into plastic seedling trays (5 cm diameter x 5.5 cm) containing 0.08 L of different types of soils and either infected with *R. necatrix* using wheat grains (three grains per slot) or not infected to be used as controls. Three sets of fifty wheat seedlings were tested per type of soil. The seedlings were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms were evaluated, and the disease index percentage was calculated as previously described for the avocado/*R. necatrix* system (Cazorla *et al.*, 2006). Disease index percentage was recorded after evaluation of symptoms, with

values ranging between 0 (healthy plant), 1 (yellowing stem base), 2 (drying stem base), and 3 (dead plant). The number of diseased seedlings was determined 7 weeks after beginning the assay, and the disease index was calculated as previously described (Cazorla *et al.*, 2006).

2.6 Soil DNA extraction

Soil DNA extraction was performed using 1.0 g of soil samples and a PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc, Carlsbad, CA, USA). DNA was extracted from three independent soil samples per pair of trees for amended and unamended soil (AS and CT) and checked for quality. To test the DNA quality we performed a DNA digestion using the restriction enzyme *Eco*RI (New England BioLabs®, Inc, Ipswich, MA, UK) and PCR amplification of the variable region of the bacterial 16S rDNA with the universal bacterial primers 341F and 907R as described by Muyzer *et al.* (2004). Digestion and PCR products were analysed for size by agarose gel electrophoresis and ethidium bromide staining. Suitable subsamples were mixed and DNA quantity and quality (A_{260}/A_{230} >1.8 and A_{260}/A_{280} >1.7) were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Three independent DNA extractions were performed per each pair of trees, and then merged to create a composite DNA sample. Three of these composite DNA extractions were independently analyzed for each type of field soil (AS and CT). DNA was stored at −20◦C for further analyses.

2.7 Analysis of 16S rRNA and ITS gene sequence

Two composite DNA samples from each soil type were sent for sequencing by STAB VIDA (NGS Laboratories, Caparica, Portugal) and sent to ChunLab (Seoul, Korea) to obtain the microbial DNA sequences of the 16S rRNA gene and ITS hypervariable

regions. Sequences were analyzed using QIIME software (Caporaso *et al.*, 2010) and CLcommunity™ software (ChunLab). Sequences of a length less than 200 nt were excluded from the analysis. The data were filtered for noisy sequences, checked for the presence of chimeras, and binned into OTUs (Peiffer *et al.*, 2013) at the 97% sequence similarity level. A representative sequence of each OTU was taxonomically classified. The relative abundance of microbial clades at different taxonomic levels was calculated as the average value from two independent analyses and was used to perform the comparative distribution analysis.

2.8 GeoChip® analysis

Three of the composite samples of purified test DNA (800 ng per sample) from the two different types of soils studied (AS and CT) were sent to Glomics Inc (Norman, Oklahoma) for the sequencing analysis (Tu *et al.*, 2014). Briefly, after the hybridization steps, the arrays were washing, dried and then scanned. The images obtained were analyzed by NimbleScan software (Roche NimbleGen Inc., Madison, WI) using the gridding file containing GeoChip® 4.6 probes and NimbleGen control probes to determine the intensity of each spot and to identify low quality spots, which were removed prior to statistical analysis (probe spots with coefficient of variance > 0.8 were removed). Extracted data were then loaded into the GeoChip data analysis pipeline at the Institute for Environmental Genomics (Microarray Data Manager, http://ieg.ou.edu/microarray/; Liang *et al.*, 2010; Deng and Zhou, 2013). First, the average signal intensity of the common oligo reference standard (CORS) was calculated for each array, and the maximum average value was applied to normalize the signal intensity of samples in each array. Second, the sum of the signal intensity of the samples was calculated for each array, and the maximum sum value was applied to normalize the signal intensity of all of the spots on an array, which produced a normalized value for each spot in each array. Spots were scored as positive based on a

floating signal-to-noise ratio [SNR = (signal mean– background mean)/background standard deviation] so that hyperthermophile control probes accounted for 5% of positive probes. Spots that were detected in less than two samples were also removed. Before statistical analysis, logarithmic transformation was carried out for the remaining spots, and the signals of all spots were transformed into relative abundances (the sum of the number of hybridized probes for each gene category or gene function between the number of total detected probes). Data processing was used for further analyses. Genes that overlapped between treatments (AS and CT) were calculated by dividing the number of overlapped genes between the treatments by the number of all genes detected in both treatments. Gene function diversity was calculated using the Shannon-Weiner index (H', alpha diversity) and Simpson's index (1/D, beta diversity). We performed a detrended correspondence analysis (DCA) to measure the differences of community functional gene structure between treatments. For comparing the different gene function communities, a hierarchical clustering analysis using Bray-Curtis distances was also performed. To analyse the unique detected probes in the AS samples, we performed a Venn diagram analysis using an on-line tool (http://bioinfogp.cnb.csic.es/tools/ venny/). Previously, we prepared two databases by selecting genes (probes) that hybridize exclusively in each type of soil and compared them. This website provided us with a list of 2766 AS unique detected sequences from suppressive soil, which were selected to perform specific comparative analysis.

2.9 Statistical methods

For suppressive analysis, the data were statistically analysed using an analysis of variance (Sokal and Rohl 1986), followed by Fisher's least significant difference test (p = 0.05) using SPSS 22 software (SPSS Inc., Chicago). For GeoChip® 4.6 analysis, significant differences in relative abundances of the microbial gene diversity between different soils were analysed by an unpaired Student's t-test. A significance level of

$p<0.1$ was adopted for all comparisons. Based on the standard error, the 95% confident interval for each response variable was obtained and the significant differences between the soils were estimated.

3. Results

3.1 Characteristics of avocado field soils

The soil samples were taken from the same avocado orchard but from trees under different soil management (AS-amended or unamended). Soil characteristics of the experimental avocado field revealed sandy-loam textures for the amended (AS) and unamended (CT) soils. The pH was not substantially different among these samples and ranged from 7.20 to 7.55 (nearly neutral pH). Some macro- and micronutrients, such as potassium, iron and manganese, were also increased in the AS-amended soil (Table 1).

3.2 White root rot suppressiveness assay

Suppressiveness assays against white root rot were performed using the avocado/*R. necatrix* and the wheat/*R. necatrix* experimental plant test systems. AS-amended and unamended avocado agricultural soils, after different experimental heat treatments an complementantions were use (Figure 5; Table 2).

Bacterial and fungal counts of AS-amended and CT soil were very similar, with values of 6.5 and 6.6 $\log_{10}$ bacterial cfu/g, respectively, and 5.0 and 5.1 $\log_{10}$ fungal cfu/g, respectively. After the heat treatment of the soil, bacterial counts decreased and stabilized, without any further changes after a second heat treatment in any type of soil (Table 3). There were no differences in the results obtained for fungal count (Table 3).

Table 3: Plate counts of total heterotrophic bacteria and fungi during the soil heat-treatment of the unamended and amended with composted almond shells. T_{0-3} indicates sampling points used along the process. Microbial counts data are presented as log10 cfu/g soil ± standar deviation.

Plate counts of	Soil source sample	Sampling points during the heat-treatment process			
		T_0	T_1	T_2	T_3
Heterotrophic bacteria	AS	6.5±0.48	5.9±0.76	6.0±0.42	5.9±0.59
	CT	6.6±0.30	5.9±0.64	5.9±0.30	5.7±0.64
Heterotrophic fungi	AS	5.0±0.90	4.7±0.67	4.9±0.57	4.7±0.60
	CT	5.1±0.98	4.9±0.55	5.0±0.67	4.8±0.87

For avocado/*Rosellinia* test system, the disease incidence was evaluated after five weeks and at the end of the assay, the disease index (DI) was calculated (Figure 6A). In these studies, AS field soil samples displayed better suppressive ability than CT field soil samples. Plants growing in the presence of AS-amended soil samples displayed a significantly lower DI than plants cultivated in the presence of CT soil samples at the end of the experiment (Figure 6A). The disease suppressiveness activity was reduced when AS soil samples were heat-treated (ASt) but showed no changes in CTt soil. Moreover, suppressiveness was complemented by soils ASc and CTt+AS, when incorporating AS soil samples. Complemented soil ASt+CT and CTc did not have a disease-suppressive ability, with levels resembling those for the heat-treated unamended soil (Figure 6A).

For the wheat/*R. necatrix* plant test system, disease incidence was tested seven weeks after inoculation when the disease index (DI) was calculated (Figure 6B). Similar to the results shown by the avocado/*R. necatrix* test system, the AS-amended soil exhibited better suppressive ability than CT soil. The suppressiveness phenotype was significantly lost in heat-treated soils (ASt and CTt) and was partially recovered when we used amended field soil to complement (ASc and CTt+AS). The soils complemented with unamended soil, CTc and ASt+CT, had a disease-suppressive ability similar to that of heat-treated unamended soil (Figure 6B).

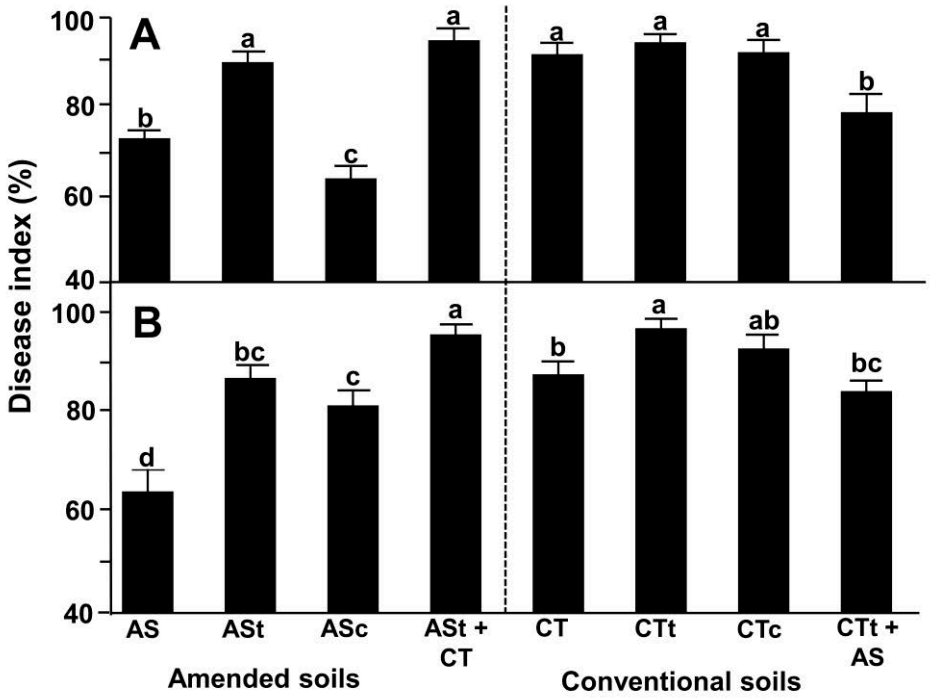


Figure 6: Suppressiveness assays using the avocado/*R.necatrix* **(A)** and wheat/*R.necatrix* **(B)** test systems.
AS: agricultural field soil amended with composted almond shells; ASt: AS heat-treated soil; ASc: ASt complemented with AS in 9:1 (w/w) ratio; ASt+CT: ASt complemented with CT in 9:1 (w/w) ratio; CT: Agricultural field soil under conventional management; CTt: CT heat-treated soil; CTc: CTt complemented with CT in 9:1 (w/w) ratio; CTt+AS: CTt complemented with AS in 9:1 (w/w) ratio.
Data were analysed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test (p=0.05). Values of bars with different letters indications denote a statistically significant difference.

3.3 Characterization of the soil microbial community based on 16S rRNA gene and ITS sequencing

DNA profiling approaches and the sequencing of 16S rRNA and the ITS variable regions of extracted and mixed DNA revealed the relative abundances of microbial clades at different taxonomic levels. However, only the most abundant OTUs were

quantified with a level of precision sufficient to perform the comparative distribution analysis due to the high level of OTU richness.

In both samples, *Archaea* were found in a very low relative abundance (<0.1%). Thus, the bacterial 16S rRNA gene sequences allowed us to identify 33 different representative phyla in AS soil samples and 26 phyla in CT soil samples, from which 5 and 7 phyla comprise more than 1% of the community in AS and CT, respectively (Figure 7).

In AS soil samples, the 5 most abundant phyla (above 89% of relative abundance) were *Proteobacteria* (50.08%), *Acidobacteria* (22.64%), *Bacteroidetes* (8.05%), *Planctomycetes* (4.27%) and *Actinobacteria* (4.09%). In contrast, the analysis of CT soil samples revealed that the most abundant (representing above 95%) phyla were *Proteobacteria* (45.48%), *Acidobacteria* (27.39%), *Bacteroidetes* (8.79%), *Planctomycetes* (60.99%), *Actinobacteria* (3.19%), *Nitrospirae* (1.70%) and *Gemmatimonadetes* (1.63%).

At the class level, the AS soils presented a high abundance of uncultured bacteria from the groups of *Acidobacteria* (EU686603, 18.44%), *Gammaproteobacteria* (17.85%), *Alphaproteobacteria* (15.28%) and *Betaproteobacteria* (11.4%) (Figure 7). In CT soil samples, the class analysis resulted in a similar representation of class abundance, including uncultured bacteria EU686603 (22.99%), *Alphaproteobacteria* (17.7%) and *Gammaproteobacteria* (10.7%).

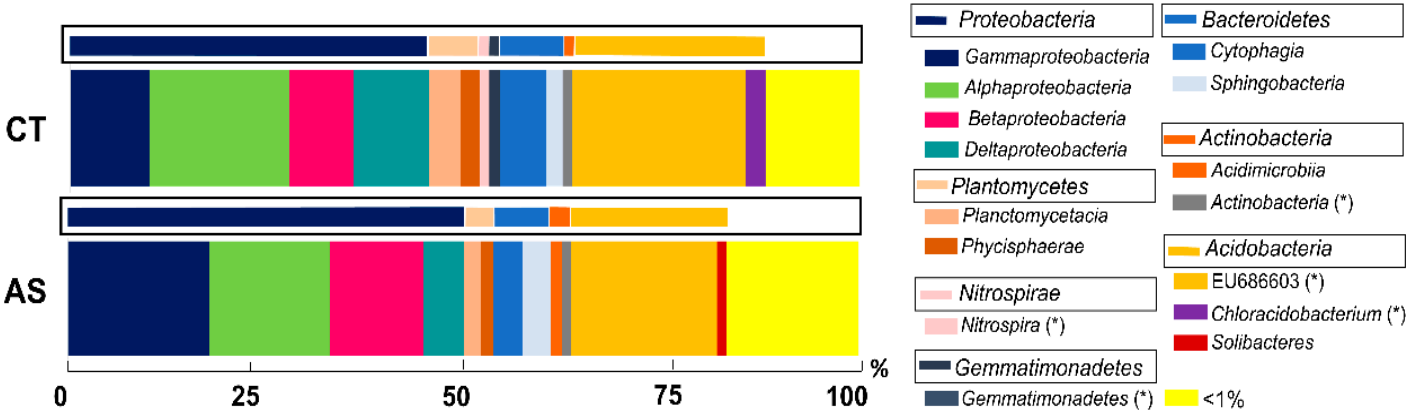


Figure 7: Analysis of microbial communities present in field soil samples unamended (CT) and amended with composted almond shells (AS). Relative abundance (percentage) of different prokaryotic groups detected by 16S rRNA gene sequencing analysis of soil DNA. Analysis of microbial groups are marked at the class level (thick bars) and the phylum level (boxed thin bars). < 1%, sum of all detected groups with a relative abundance less than 1%. *: taxonomic characteristics of these groups are uncertain.

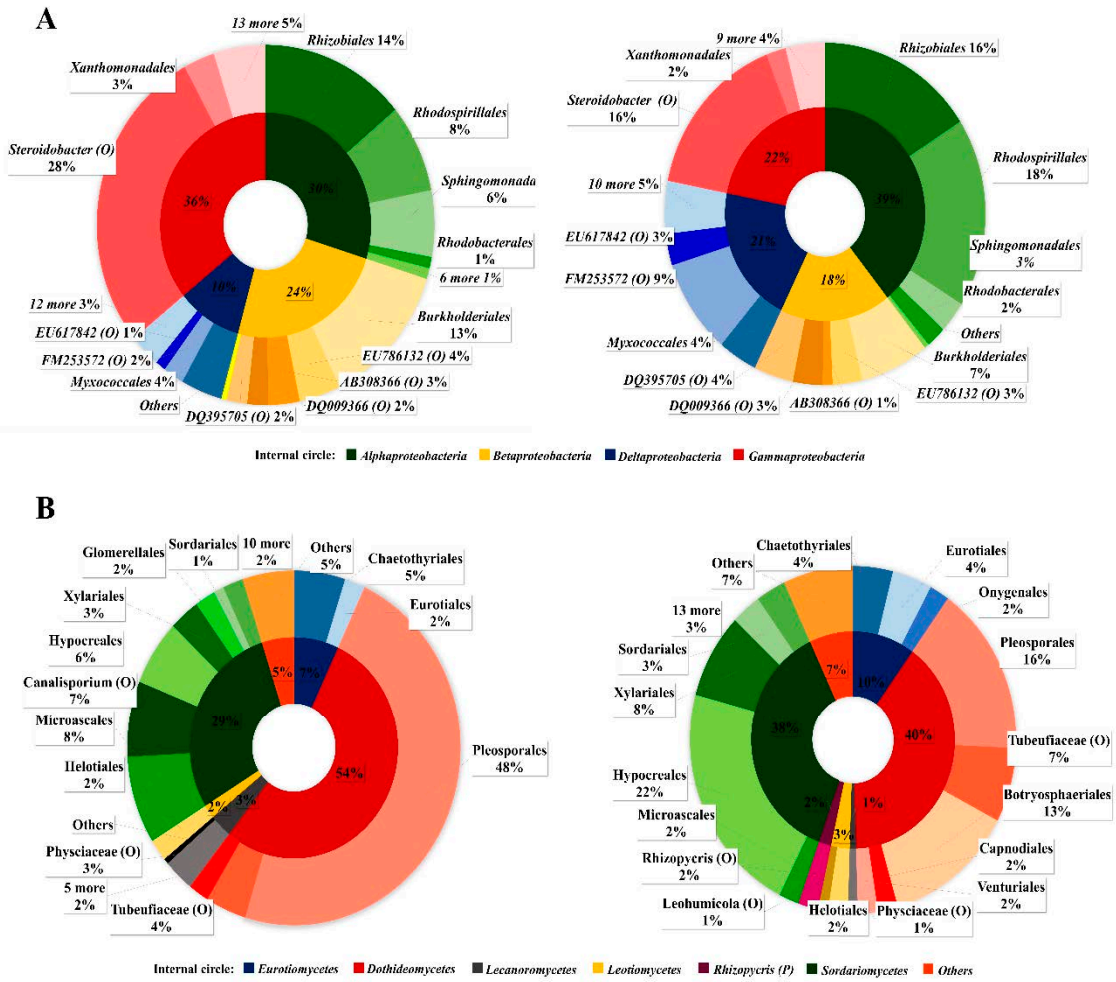


Figure 8: Microbial community analysis of most represented phyla in each samples. **(A)** Relative abundance (percentage) of different *Proteobacteria* classes (internal circle) and orders (external circle) detected by 16S rRNA gene sequence analysis of soil DNA isolate from amended soil (AS) or conventional soil (CT); **(B)** Relative abundance (percentage) of different *Ascomycota* classes (internal circle) and orders (external circle) detected by ITS region sequence analysis of soil DNA isolate from amended soil (AS) or conventionally managed soil (CT).

In both soil samples, the phylum *Proteobacteria* is the most abundant (50.08% and 45.48%). Differences in this group have been shown between the two soil samples. In general, diversity is higher in AS soil samples that exhibit a predominance of the classes *Gammaproteobacteria* (36%) and *Alphaproteobacteria* (30%) and a low percentage of *Deltaproteobacteria*. In CT soil samples, a clear predominance of *Alphaproteobacteria* can be observed (39%). Remarkably, we observed an increase in AS soil samples (almost 2x) of the orders *Steroidobacter* (28%) and *Burkholderiales* (13%) and the decrease of *Rhodospirales* (from 18% in CT to 8% in AS) (Figure 8A). We observed 76 different classes in AS soil samples and 65 classes in CT soil samples. We detected 24 and 13 specific bacterial classes in AS and CT, respectively, and a slightly higher richness in AS samples (Figure 9A).

The analysis of ITS sequences to reveal the abundance of eukaryotic microbes allowed us to identify a high abundance of fungal microbes. Eukaryotic microbes different from fungi ranged from 7.97% (AS) to 9.52 (CT). Among the fungi detected, the unclassified fungi comprises 8.04% (AS) and 4.28% (CT), and those below 1% represent 2.9% in CT soil samples and 3.4% in AS soil samples.

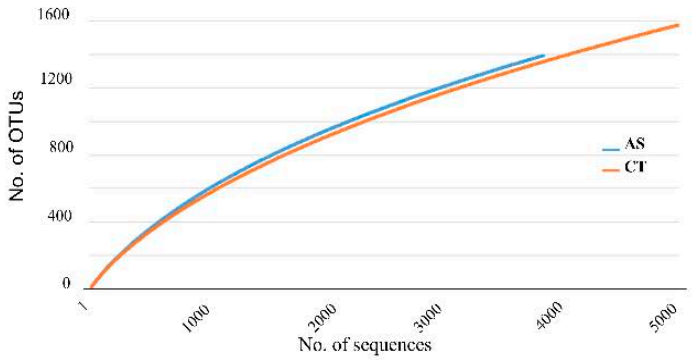
The most abundant fungal groups (approximately 70%) that are in both soil samples are of the phyla *Ascomycota* and *Basidiomycota* and of the group *Mortierellales*. In AS soil samples, an increase in the relative abundance of *Ascomycota* can be observed (Figure 10), (35.37% in CT and 45.79% in AS), as well as a reduction in the group of *Mortierellales* (18.37 in CT and 9.92% in AS).

A

16S rRNA	Valid reads	OTUs	Chao1	Coverage (%)	Shannon (H')	Simpson (D)
AS	3888	1396	2653.34	79.84	6.63	0.0026
CT	5046	1578	3037.59	82.46	6.57	0.0035

B

ITS	Valid reads	OTUs	Chao1	Coverage (%)	Shannon (H')	Simpson (D)
AS	6142	664	940.13	96.12	5.27	0.012
CT	7288	787	998.53	96.67	5.33	0.017



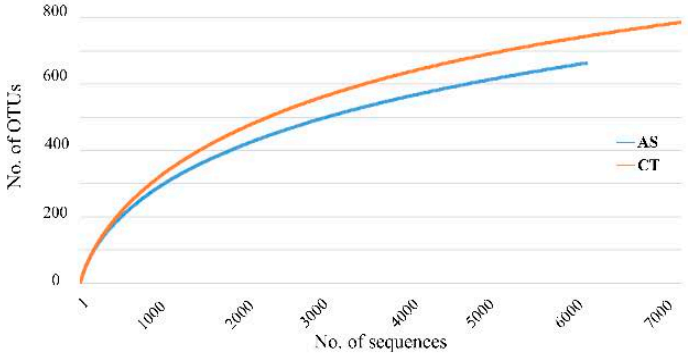


Figure 9: Quality indixes, alpha-diversity and rarefaction curve of sequencing analysis**. (A)** Quality indixes (valid reads, OTUs, Chao1 and coverage %) richness (Shannon) , eveness (Simpson) and rarefaction curve obtained of 16S rRNA gene sequence analysis of soil DNA isolate from amended soil (AS) or conventional soil (CT); **(B)** Quality indixes (valid reads, OTUs, Chao1 and coverage%) richness (Shannon) , eveness (Simpson) and rarefaction curve obtained of ITS region sequence analysis of soil DNA isolate from amended soil (AS) or conventionally managed soil (CT).

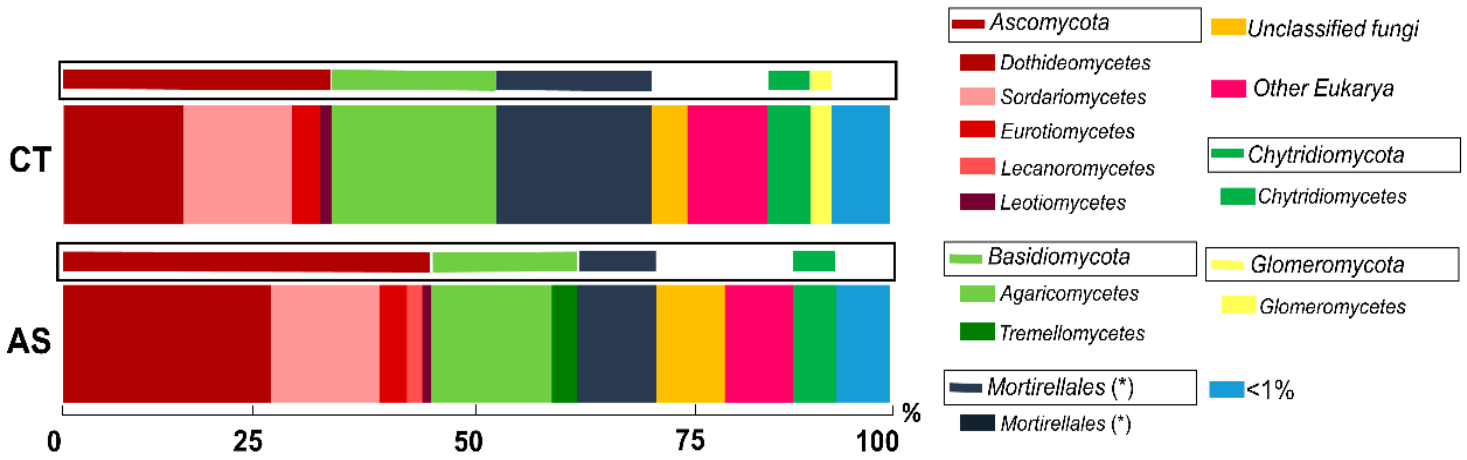


Figure 10: Analysis of microbial communities present in field soil samples unamended (CT) and amended with composted almond shells (AS). Relative abundance (percentage) of different eukaryotic groups detected by ITS region sequence analysis of soil DNA. Analysis of microbial groups are marked at the class level (thick bars) and at phylum level (boxed thin bars). < 1%, sum of all detected groups with a relative abundance less than 1%. *: taxonomic characteristics of these groups are uncertain.

The analysis of the most abundant group of microorganisms (*Ascomycota*) revealed that in AS soil samples an increase of the class of *Dothideomycetes* (from 40% in CT to 54% in AS) was observed (Figure 10). Additionally, a reduction of the class of *Sordariomycetes* (from 38% in CT to 29% in AS) was observed. Also of note in reference to fungal order in AS soil samples, a huge increase of *Pleosporales* (from 16% in CT to 48% in AS) was observed. Remarkably, one of the fungal order that decreased in AS soil samples was the order *Xylariales* (from 8% in CT to 3% in AS), where the pathogen *R. necatrix* is allocated (Figure 8B).

We observed 39 different classes in AS soil and 50 classes in CT soil. We detected 7 and 18 specific bacterial classes in AS and CT soil, respectively, and observed a slightly higher richness in CT samples (Figure 9B).

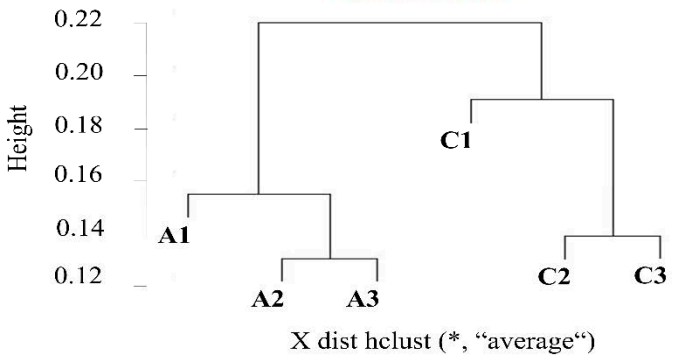
3.4 GeoChip® analysis in soil samples

The number of total genes detected by GeoChip analysis and overlapping genes between treatments were measured to understand the functional diversity and structure of the microbial communities. The number of total genes detected ranged from 27348 to 28491 and from 29311 to 33526 in AS and CT samples, respectively. An unpaired Student's t-test showed that these values were significantly different. The percentage of overlapping genes between samples ranged from 77.18% for AS (77.41%, 75.25%, and 78.88%) to 73.16% for CT (76.25%, 65.70%, and 77.52%) (Figure 11A). This value fell to 65.43% when we compared overlapping genes between treatments (AS_{1-3} and CT_{1-3}). DCA (detrended correspondence analysis) and hierarchical clustering (with Bray-Curtis distance) were performed (Figure 11B-C) using all of the detected genes, showing that functional structure of the microbial community was similar in the replicates but different among the soils (AS and CT).

A

	AS1	AS2	AS3	CT1	CT2	CT3
AS1	-	24364 (77.41)	23686 (75.25)	22665 (66.67)	24157 (65.79)	21790 (61.69)
AS2			24829 (78.88)	23074 (66.44)	25405 (69.39)	22920 (64.86)
AS3				22908 (66.95)	24545 (66.71)	21671 (60.35)
CT1					27185 (76.25)	23424 (65.70)
CT2						27639 (77.52)
CT3						-
Num. of detected probes (*)	27348	28491	27813	29311	33526	29765

B



C

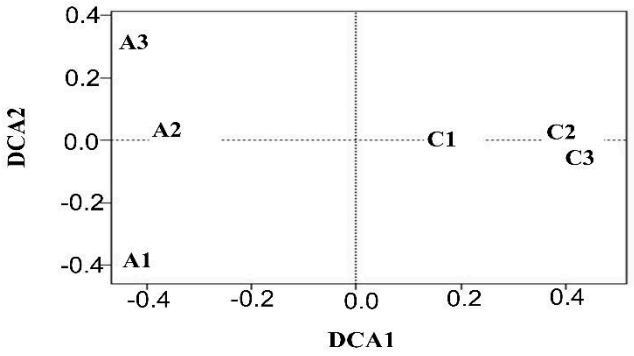


Figure 11: Unique and overlapped genes, diversity indexes and beta-diversity from GeoChip analysis. **(A)** Summarized data of GeoChip analysis**:** italicized values indicates the number of overlapping genes between samples; values in parentheses shown percentages of overlapping genes between samples; (*) significantly different. **(B)** Bray-Curtis average distance cluster dendogram. **(C)** Detrended correspondance analysis (DCA) of samples from amended soil and convetionally managed soil treatments.

To understand the effects of composted almond shells on the microbial communities and the acquired suppressive capacity, microbial functional genes categorized as participating in biogeochemical cycles and other important soil processes were examined (Figure 12). Gene functions related to the carbon cycle were the gene category most represented in all samples. C cycling probes were significantly more abundant than other categories in AS samples (36.65% in AS and 34.54% in CT), whereas genes related to organic contaminant degradation (12.42% in AS and 12.81% in CT), metal resistance (14.58% in AS and 16.32 in CT) and virulence (1.59% in AS and 1.61% in CT) were significantly more abundant in CT samples. There were no significant differences in N, P and S cycle genes and other gene categories such as stress, fungi functions, soil benefit and soilborne pathogens (Figure 12).

Key genes for acetogenesis, C degradation, C fixation, methane metabolism and other genes related to the C cycle were detected in the two types of soils (Figure 13A). The relative abundance of genes related to the C degradation category were the highest and exhibited significant differences between the AS samples and the CT samples. In this category, we found the presence of degradative genes of the most abundant C sources derived from plant and animal sources that could be present in soil ecosystems, such as starch, hemicellulose, cellulose, chitin and lignin. There were few significant differences between samples in these categories of detected genes (Figure 13A).

Of the nitrogen cycle category, only the ammonification subcategory had a higher significant difference for amended soil (Figure 13B). In this subcategory, there are genes that function in the decomposition of organic matter and cycling of accumulated N source.

Related to the sulphur cycle, the analyses performed exhibited a higher significant difference (p<0.1) in only the sulphite reductase genes of AS samples compared to CT samples. These genes encode enzymes that catalyse the reduction of sulphite to sulphide, using iron as cofactor, and provide a source of S to microbiota.

.

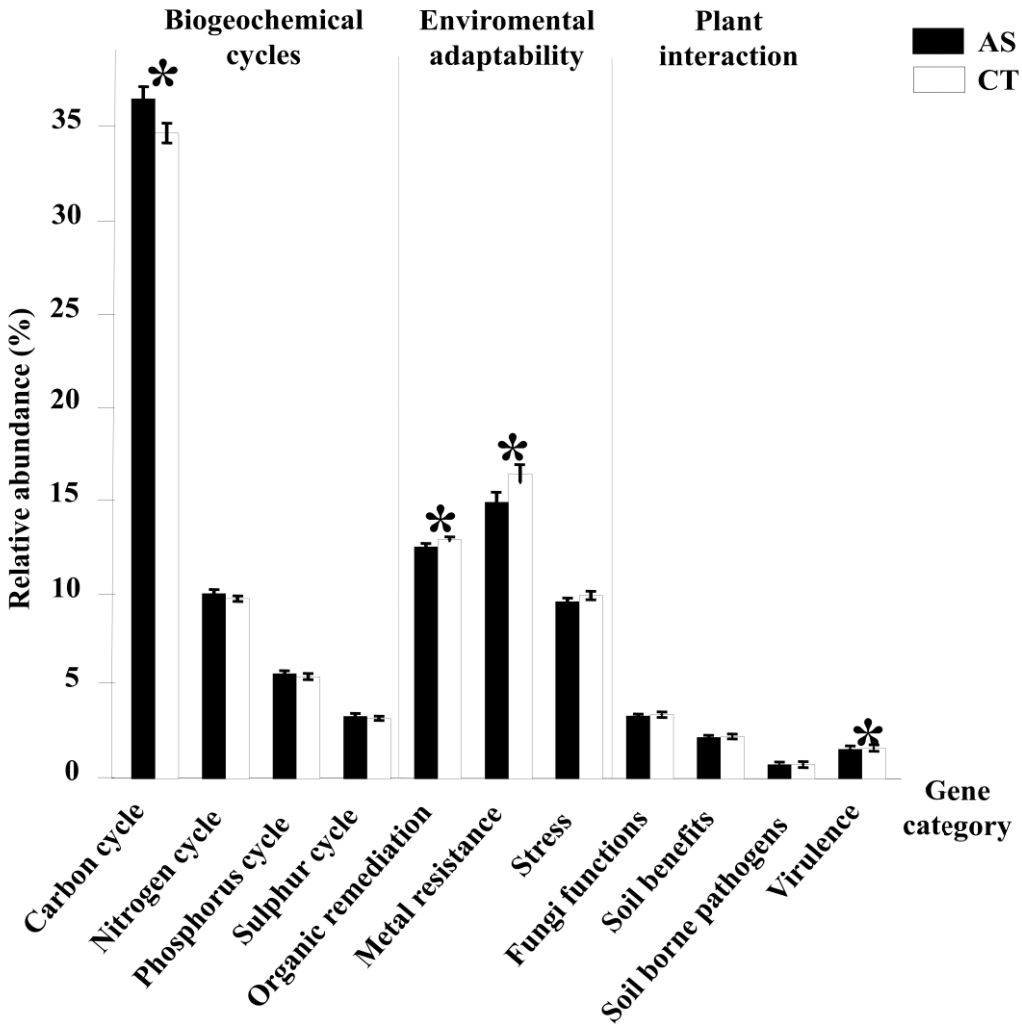


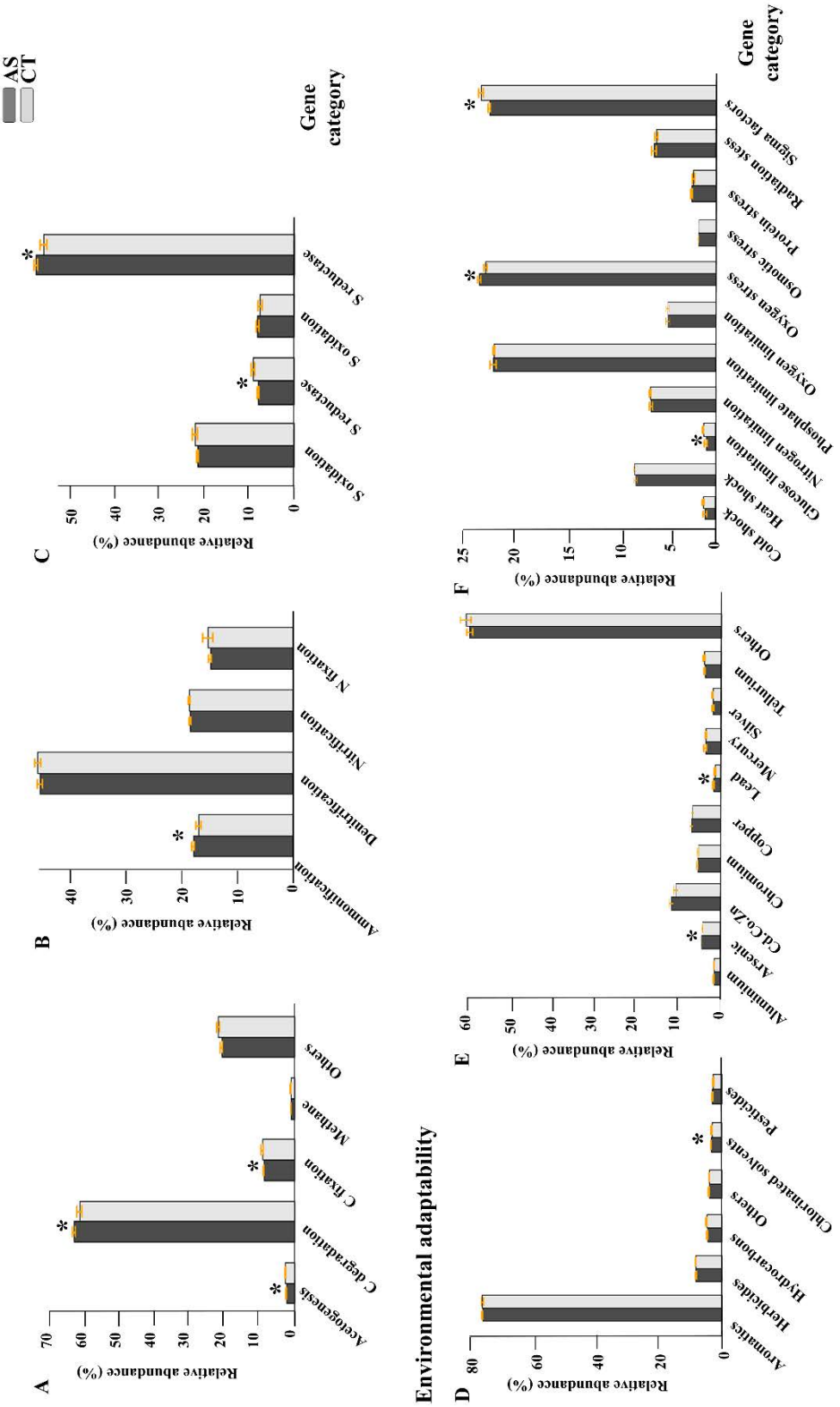
Figure 12: GeoChip analysis of functional gene categories. Relative abundance of all detected genes from different gene categories analysed in this study. * indicates significant statistical differences (p<0.1) between the two types of soil samples, amended soil (AS) and conventionally managed soil (CT). Standard deviation bars are shown.

The CT samples exhibited a higher significant difference in sulphate reductase, a protein involved in sulphur reduction by anaerobic respiration (Figure 13C).

Statistical analyses showed no significant differences in the relative abundance of genes involved in the phosphorous cycle for these samples.

The analysis of genes in the category of environmental adaptability showed significant differences (p<0.1) in the subcategories, as shown in Figure 13D-F. Genes involved in the organic degradation of aromatics, such as chlorinated and pesticide-related compounds, had a higher significant relative abundance for amended soil than conventional managed soil. Similar results were obtained for genes related to osmotic and oxygen stress, from the stress category, and metal resistance to cobalt and lead, which had slightly higher significant relative abundance for AS samples than CT samples. On the other hand, unamended soils exhibited significantly higher values of relative abundance for genes related with stress induced by glucose limitation and metal resistance to cadmium and other metals.

The category of plant interaction covers a wide range of different functional genes involved in microbial interactions with plants, including genes related to fungal function, soil benefit, soilborne pathogens and virulence. The analyses performed showed significant differences (p<0.1) in some subcategories, as shown in Figure 13G-J. There were not any significant differences in the genes in the categories of soil benefit or fungi function. Nevertheless, CT samples exhibited a higher significant relative abundance of detected genes from the oomycetes subcategory (soilborne pathogen), which included different genes from this pathogenic group. Genes related to virulence processes such as iron oxidation or secretion had a higher significant relative abundance for amended soils; whereas unamended soils exhibited significantly higher values for genes involved in virulence actions such as iron uptake (aerobactin genes) and pilin formation



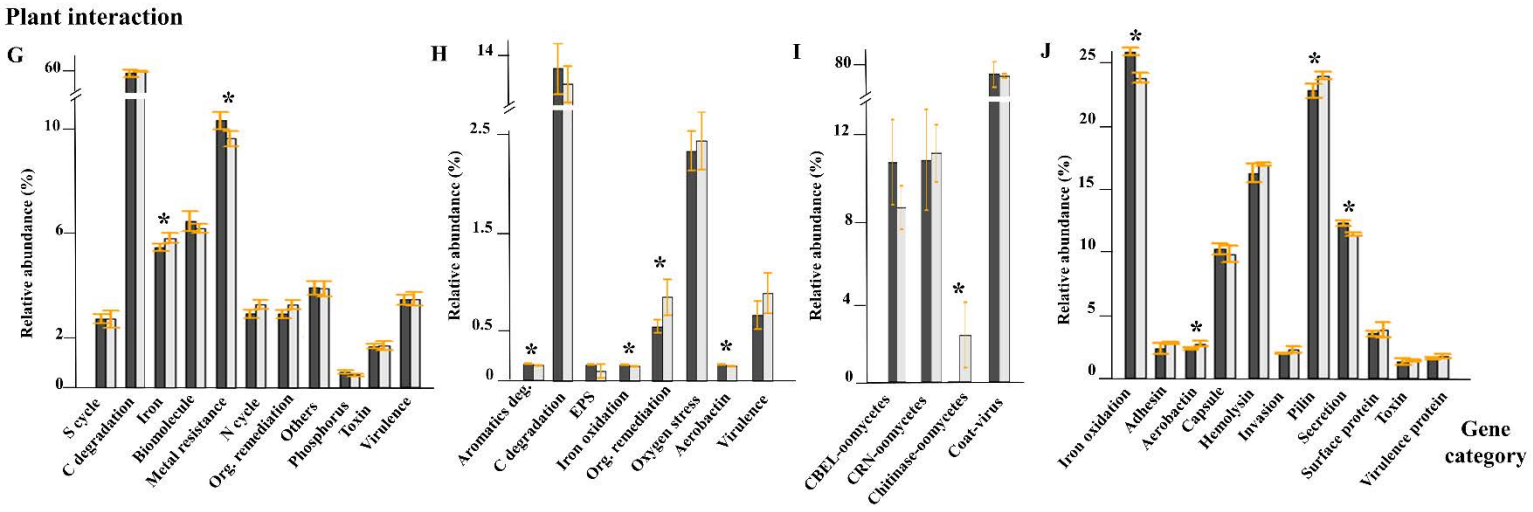


Figure 13: Biogeochemical cycles analysis. (**A)** Relative abundance of detected genes from Carbon cycle gene categories **(B)** Relative abundance of detected genes from Nitrogen cycle gene categories **(C)** Relative abundance of detected genes from Sulphur cycle gene categories; **Enviromental adaptability analysis. (D)** Relative abundance of detected genes from different gene categories related with organic compounds degradation **(E)** Relative abundance of detected genes from different gene categories related with metal resistance. **(F)** Relative abundance of detected genes from different gene categories related with stress situations; **Plant interaction analysis. (G)** Relative abundance of detected genes from different gene categories related with fungi functions **(H)** Relative abundance of detected genes from different gene categories related with soil benefits compounds. **(I)** Relative abundance of detected genes from different gene categories related with soil borne pathogens. **(J)** Relative abundance of detected genes from different gene categories related with virulence activity. Asterisc show significant stastitical differences (p<0.1) among the two types of soil samples, amended soil (AS) and conventionally

3.5 Unique DNA probes detected in AS suppressive soil samples

Results of the GeoChip analysis and the Venn diagram representation allowed us to determine microbial specific gene functions detected exclusively in each treatment and the number of commonly detected probes (27364) (Figure 14A). We found 6674 unique detected probes in CT samples and 2766 unique detected probes in AS samples (approximately 10% of the total AS detected genes) from the gene categories analysed. Approximately 34.49% of the unique hybridizations were related to the Carbon cycle category (Figure 14B), mainly to starch and chitin degradation (Table 4). The Organic remediation gene category exhibited 14.53% unique hybridizations of genes related to the degradation of aromatic compounds. The Stress category had 13.38% unique hybridized probes and the Metal resistance category had 11.86% unique hybridized probes. The Nitrogen cycle category exhibited 8.57% unique hybridized probes, mostly in genes related with denitrification. The remaining gene categories had lower percentages: Sulphur cycle 5.60%, Fungi function 3.69%, Soil benefit 2.64% (approximately 44% of unique detected probes in this category correspond mainly with antimicrobial genes such as *cat* (catalase), *phz*F (phenazine) or *pcb*C (isopenicillin)), Phosphorus cycle 2.28%, Virulence 1.88% and Soil borne pathogen 1.08% (Figure 14B). This analysis allowed us to relate different gene functions implicated in the metabolism of different soil compounds with bacterial or fungal classes present in the AS soil (Table 4).

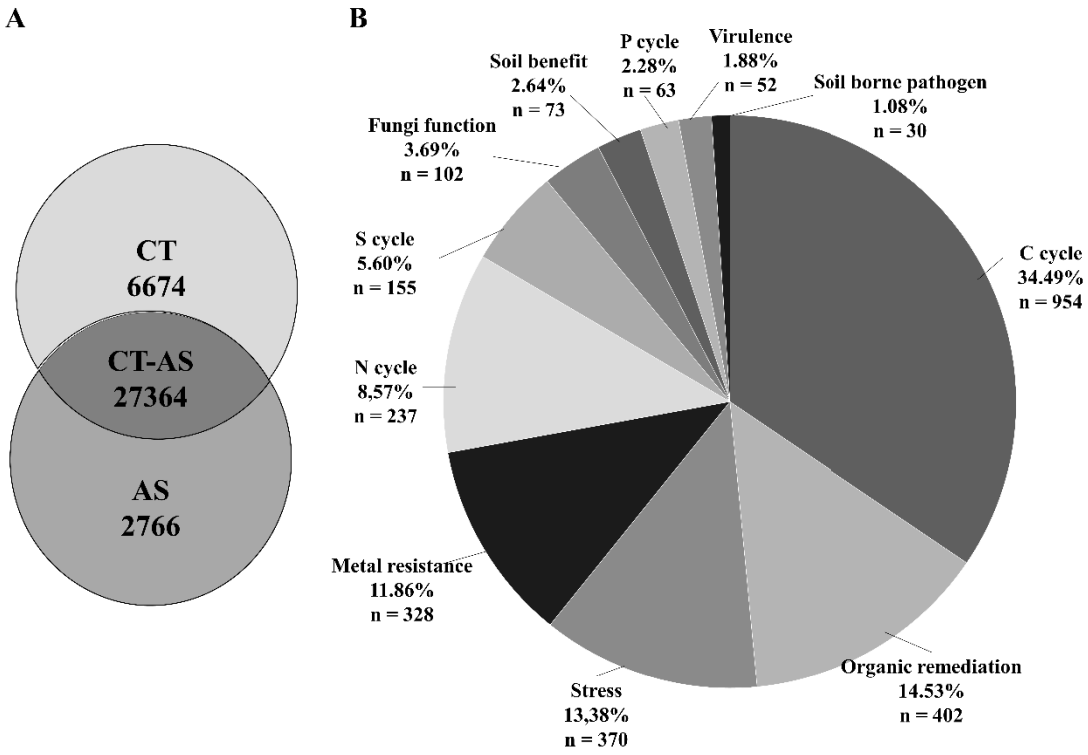


Figure 14: GeoChip analysis of unique detected genes. **(A)** Number of core and unique detected genes (different gene ID) of amended soil (AS) and conventionally managed soil (CT) **(B)** Assigned functions of the unique genes detected in the AS sample.

Table 4: Assigned function and identity (lineage at class level) of the unique genes detected in AS sample. Classes (bacterial and fungal) related with functional gene categories of unique detected genes of amended soil (AS).

Gene Category	Class		R.A (%)
Carbon cycling	**Bacteria**	**Fungi**	**34.49**
Carbon fixation	*Actinobacteria; Bacteroidia; Betaproteobacteria*		
Starch	*Thermoprotei; Thermoccoci; Thermoplasmata; Actinobacteria; Bacteroidia; Chlamydiia; Chrococcaceae; Bacilli; Clostridia; Deltaproteobacteria; Gammaproteobacteria; Synergistia; Thermotogae*		
Hemicellulose	*Bacilli; Clostridia; Gammaproteobacteria*		
Cellulose	*Bacilli; Clostridia; Betaproteobacteria*	*Neocallimastigomycetes*	
Chitin	*Bacilli; Gammaproteobacteria*		
Lignin		*Agaricomycetes; Dacrymycetes*	
Others Carbon cycling	*Bacilli; Negativicutes; Fusobacteriia; Betaproteobacteria; Epsilonproteobacteria; Gammaproteobacteria*	*Orbiliomycetes; Saccharomycetes; Sordariomycetes*	
Organic remediation			**14.53**
Aromatic carboxiyic acids	*Actinobacteria; Dehalococcoidetes; Bacilli*		
Herbicides related compounds	*Clostridia; Alphaproteobacteria; Betaproteobacteria; Deltaproteobacteria*		
Others aromatics	*Gammaproteobacteria*	*Saccharomycetes*	
Stress			**13.38**
Oxygen stress	*Gammaproteobacteria*		
Phosphate limitation stress	*Bacilli; Clostridia; Alphaproteobacteria;*		
Heat shock	*Sphingobacteriia*		
Osmotic stress	*Deferribacteres*		

(continuation Table 4)

Gene Category	Class		R.A (%)
Metal Resistance	**Bacteria**	**Fungi**	**11.86**
Copper Tellurium Arsenic Chromium Others Metal resistance	*Bacilli* *Alphaproteobacteria; Gammaproteobacteria;* *Betaproteobacteria;* *Gammaproteobacteria; Mollicutes* *Methanobacteria; Actinobacteria;* *Dehalococcoidetes; Clostridia;*		
Nitrogen			**8.57**
Ammonification Denitrification Nitrogen fixation Nitrogen cycle others	*Bacilli* *Bacilli; Alphaproteobacteria;* *Clostridia* *Epsilonproteobacteria; Deltaproteobacteria;* *Spirochaetia;*		
Sulphur			**5.60**
sulfite reductase	*Acidobacteria; Alphaproteobacteria;* *Epsilonproteobacteria; Deltaproteobacteria;* *Gammaproteobacteria*	*Coscinodiscophyceae*	
Fungi function			**3.69**
Metal resistance Fungi Carbon degradation Lignin		*Eurotiomycetes* *Oribiliomycetes; Saccharomycetes;* *Sordariomycetes;* *Agaricomycetes; Dacrymycetes*	
Soil benefit			**2.64**
Antimicrobial	*Actinobacteria; Bacilli; Clostridia;* *Gammaproteobacteria*	*Sordariomycetes*	
Phosphorus			**2.28**
Phosphorus utilization	*Nostocaceae; Epsilonproteobacteria;* *Gammaproteobacteria;*		
Virulence	*Gammaproteobacteria*		**1.88**
Soil borne pathogen	*Gammaproteobacteria; Tombusviridae;* *Virgaviridae; Secoviridae*	*Sordariomycestes*	**1.08**

R.A. relative abundance.

4. Discussion

The application of organic amendments to agricultural soils is a longstanding practice, and examples of organic-amendment-mediated suppression of soilborne diseases were reported as early as the late 19th century (Stone *et al.*, 2004). At present, nursery and greenhouse growers successfully use compost-amended potting mixes to suppress soilborne diseases, such as *Pythium* and *Phytophthora* root rots, in container systems

(Hoitink *et al.*, 1991). However, limited field studies have been conducted to determine the impact of soil amendments on microbial communities in actual organic and conventional production systems (Drinkwater *et al.*, 1995; Gunapala and Scow, 1998; Bulluck and Ristaino, 2001). In the case of avocado orchards, organic matter-mediated disease suppression against *Phytophthora cinnamomi* has been observed in avocado agricultural fields organically managed in Australia. Organic amendments (barley straw, sorghum residues, and native grass) were added to the soil under the trees as a mulch layer resulting in the suppression of *Phytophthora* root rot of avocado (Malajczuk, 1979; 1983). Additionally, our previous studies also demonstrated that different organic amendments can influence the composition and diversity of soil bacterial communities in avocado plants growing in microcosms after DGGE analysis, showing enhancement of specific populations such as Burkholderia and Frateuria (Bonilla *et al.*, 2012a; 2015). Among different organic matter tested on avocado crops, composted almond shells (AS; commercial almond shells derived from the almond industry were piled and traditionally composted) exhibited enhancement of soil suppressiveness against *Rosellinia necatrix* (Bonilla *et al.*, 2012a), the causal agent of avocado white root rot (Pliego *et al.*, 2012). Even when soil suppressiveness against *R. necatrix* is improved after the addition of AS, only subtle changes in the bacterial community and composition and specific enzymatic activities have been reported using DGGE analysis (Bonilla *et al.*, 2015). It must be considered that a wide range of factors can affct soil microorganisms communities (van Veen *et al.*, 1997). The soil samples used in our study came from the same orchard (same type of soil, environmental conditions, plant age and cultivar, etc.), but were under different management, and this was assumed to be the only difference between the samples. The soil influenced by the amendment of AS showed some characteristics that differed from the conventional unamended soil. The almond shells are a high dry matter-containing substrate, composed of approximately 95% organic matter, with poor

values of glucose, fructose or sucrose, The characteristics and composition of AS makes this susbstrate an acceptable growing media for soilless culture (Valverde *et al.*, 2013). Moreover, it must be taken into account that the avocado is a shallow rooted tree, with needs good aeration. Roots are helped by the presence of rich surface of organic mulch, as shown by the tendency of healthy feeder roots to grow into any decomposing litter layer (Chanderbali *et al.*, 2013).

In this work, a metagenomics approach to the community composition of amended and unamended avocado soils have been performed for the first time. The use of metabarcoding and GeoChip techniques allowed a better knowledge on the community composition and their potential activities. In first place, an attempt to identify key factors involved in this enhanced suppressivity after the addition of organic amendments revealed the crucial role of the microbiota present in the orgaic amended soil. The microbiota evolved in the composted almond shells was crucial for suppressiveness because the reduction of the bacterial population after a heat treatment in the organic amendment resulted in a more conducive phenotype (heat-treated soil samples harbour 10^5 cfu/g, most likely composed mainly by sporulated bacterial and fungal microorganisms). Moreover, total or partial suppressiveness was recovered when these heat-treated soil samples were complemented with a portion of soil influenced by AS, but it remained conducive when complemented with a portion of conventional soil (CT). This effect has been previously described for different suppressive soils, where sterilization by autoclaving, steam pasteurization, and irradiation rendered soils conducive to the pathogen studied (Malajczuk, 1983; Weller *et al.*, 2002; Mendes *et al.*, 2011). Suppressiveness experiments performed do not excluded the possibility that the disinfected avocado root used could harbour endophytic microorganisms, but our results significantly pointed out the role of the composted almond shells in the plant protection against *R. necatrix*. Thus, our results

support the crucial role of microbes present in AS for turning the conducive CT soil into a more suppressive soil against *R. necatrix*.

To gain insights into the microbial diversity present in the soil samples, we used several different approaches. Phylogenetic marker analysis based on the sequencing of 16S rDNA and ITSs revealed a relatively similar array of prokaryotic and eukaryotic populations present in the AS and CT soil samples; however, a different response has been described in the literature for other types of organic matter from different sources, such as composted municipal waste (Zaccardelli *et al.*, 2013). It is remarkable that in our model system, the group of fast-growing, easily cultivable *Proteobacteria* is the dominant group of prokaryotes in both soil samples. These data are similar to those previously observed for other soil and rhizosphere samples with a high presence of organic matter (Lynch and Whipps, 1990; Paul and Clark, 1996; Hawkes *et al.*, 2007; Mendes *et al.* 2011). Moreover, the representation of the other phyla different than *Proteobacteria* were quite similar among AS-amended and unamended soils, thus contradicting the idea that a highly specific community is stimulated by the addition of AS. Diversity analysis confirmed the previously obtained results (Bonilla *et al.*, 2015), highlighting the enhancement of specific microbial populations in AS-amended samples, such as *Betaproteobacteria* (*Burkholderiales*) and the class of *Gammaproteobacteria*, which have been reported to protect plants from fungal infections in other suppressive soils (Mendes *et al.*, 2011). It is important to note the clear enhancement in AS-amended soil of the order *Steroidobacter*, previously reported to play an essential role in the positive interactions with plants; for example, controlling seed germination, stem and root elongation or stress protection in plants (Zarraonaindia *et al.*, 2015).

In contrast, analysis of eukaryotic ITS revealed a different abundance distribution of microbes among the two types of soil samples. Fungal clones were the most common and dominant microbial eukaryotes in the soil. AS-amended soil samples had an

increased relative abundance of *Ascomycota*. This fact is not surprising considering that *Ascomycetes* are the largest group on true fungi (Larena *et al.*, 1999). Moreover, the dominance of *Ascomycota* has been observed during different composting processes (De Gannes *et al.*, 2013; Neher *et al.*, 2013), where most of them are saprophytic and live on dead organic material that they help decompose (Agrios, 1997; Viebahn *et al.*, 2005). This behaviour easily explains their higher abundance when composted almond shells are added to the soil as a mulch. Within *Ascomycota*, the group that exhibited the most apparent and highest increase of abundance in AS-amended soil samples was the fungal class of *Dothideomycetes*. A high abundance of *Dothideomycetes* in soils with at high hydrocarbon concentrations has been previously reported (Ferrari *et al.*, 2011), suggesting its preference for those habitats with a high concentration of organic matter where it participates in biomass conversion (Shrestha *et al.*, 2011). Moreover, the large increase of the phylum *Pleosporales* (*Dothideomycetes*) is also not surprising because this group is very well known to contain species that chlorinate lignin as a first step of biomass conversion during plant litter degradation (Ortíz-Bermúdez *et al.*, 2007). Interestingly, it has been shown that several genera of *Dothideomycetes* exhibit an increased presence in suppressive soils because they harbour endohyphal bacteria from groups that are capable of hydrocarbon biodegradation, such as the *Xanthomonadales*, *Pseudomonadales*, *Burkholderiales* and *Sphingomonadales* (Hoffman and Arnold, 2010). *Dothideomycetes* have also been shown to increase slightly in AS-amended soils. However, the group that shows an apparent decrease in AS-amended soils is *Mortierellales*. This group has a complex phylogeny (Wagner *et al.*, 2013) and is considered to be ubiquitous in the bulk and rhizospheric soil, implying that it could play a role in maintenance of the microecological balance (Miao *et al.*, 2015). Interestingly, the group of Glomeromycota, which contains different groups of symbiotic fungi previously detected in avocado (Hass and Menge, 1990; González-Cortés *et al.*, 2012), it is clearly

detected in unamended soils, but decreased in the amended ones (below 1%). A possible explanation could be that in the AS amended soils, take place a strong competition with other decomposing fungi, such as the *Dothideomycetes*, more adapted to an environment with high amount of decomposing organic matter. Finally, it should be noted that members of *Xylariaceae*, to which *R. necatrix* belongs (Pliego *et al.*, 2012), are less abundant in AS-amended soils, thus revealing a negative effect on this fungal group. These results indicate that the soil fungal community was affected by the soil amendment with AS.

Phylogenetic markers such as the prokaryotic 16S and eukaryotic ITS region do not carry explicit functional information. For this, the use of GeoChip-based analysis allowed for the analysis of microbial functional genes encoding key enzymes involved in major biogeochemical processes that facilitate linking microbial community structure to potential ecological functions (Torsvik and Ovreas, 2002). Using this technique, we screened potential functional gene diversity among unamended and AS-amended soil samples.

Probe signals and DCA analysis indicated that the microbial community functional structures differed between CT and AS soil samples. The sample sites are very close together, so the differences observed in the microbial communities are thought to be the result of amendment with organic matter.

Generally, similar abundance patterns of functional genes involved in nutrient cycling processes such a nitrogen, phosphorous or sulphur cycling, were found in both types of samples. However, AS-amended samples had higher signal intensities for C degradation (carbon cycle) genes than CT, with some differences being statistically significant. Substrates for this group of genes ranged from labile C to more recalcitrant C (e.g., starch, hemicelluloses, cellulose, chitin and lignin). These results suggest that AS-amended microbial have a greater capacity for C degradation than CT communities. This suggests, as expected, an important role of carbon cycling in

response to the addition of organic matter to the soil. However, no differences in gene abundance for N, P or S cycling was observed. This can be explained because almond shells are a lignin-rich waste resulting from the almond industry, mostly composed of approximately 27% lignin and 73% holocellulose (Caballero *et al.*, 1996), and those cycles were not compromised. However, statistical differences in the abundance of genes related to organic remediation and metal resistance were observed in AS-amended soil displaying lower levels than CT. This observation may be due to a decrease in the available compounds due to the high sorption ability of the composted almond shells and derivate compounds from its degradation, which have been previously reported to be able to remove such substances from the soil (Pehlivan *et al.*, 2009).

Interestingly, both soil samples shared a core of probes corresponding to approximately 90% of the assayed sequences (27364 probes). However, approximately 10% of the total probes analysed were unique for AS-amended samples (2766 probes). When the sequence of these probes were analysed, they resulted in a very similar distribution to that previously shown for the whole GeoChip analysis, with above 34.5% corresponding to C cycling, followed by probes related to organic remediation (14.5%), stress (13.4%), metal resistance (11.9%) or the N cycle (8.6%). These results support the following previously described results: systems associated with organic matter-mediated general suppression; suppression typically occurs as a result of the activation of the indigenous microbial community (Lockwood, 1990); and suppressive activities can be generated by one to few populations of organisms (Gerlagh, 1968; Cook and Baker, 1983; Hoitink and Boehm, 1999; Weller *et al.*, 2002). Postma *et al.* (2000) found that qualitative rather than quantitative shifts in the bacterial community correlate with disease suppressiveness, and several studies indicated that mechanisms within the microbial activity of the soil are responsible for

the suppression of pathogens (Rovira and Wildermuth, 1981; Nitta, 1991; Workneh and van Bruggen, 1994; van Os and van Ginkel, 2001).

Among the specific taxa stimulated, *Pseudomonadaceae*, *Burkholderiaceae*, *Xanthomonadales* and *Actinobacteria*, harbour genera and species with activity against plant pathogenic fungi (Postma *et al.*, 2010). Additionally, it is important to note that *Pseudomonas*, *Rhizobium*, *Bacillus*, *Variovorax*, *Phyllobacterium*, and *Azospirillum*, are considered the most efficient plant growth-promoting bacteria (Bertrand *et al.*, 2001).

Sequencing of specific probes present in AS-amended soils revealed the presence in such soil samples of genes for bacterial and fungal catalases, phenazine biosynthetic genes (from *Proteobacteria*) or the presence of potential antibiotics produced by *Actinobacteria* (data not shown). Nearly all of these probes corresponded to the GeoChip category "soil benefit", where the antimicrobials from different groups were analysed. To the best of our knowledge, no probes from *Bacilli* were used, so the role of antimicrobials such as iturin or fengicins, produced by *Bacillus* spp., cannot be discussed based on our results.

It is important to note that the genus *Pseudomonas* (class *Gammaproteobacteria*) and *Bacillus* (class *Bacilli*) are two of the most prominent bacteria that can be isolated from avocado soil and rhizosphere displaying antifungal activity and plant protection against soilborne pathogens (Cazorla *et al.*, 2006; 2007; González-Sánchez *et al.*, 2010). Our results reinforce the importance of such microorganisms in the soil and root ecology of the avocado crop. These groups of microorganisms can produce metabolites, such as siderophores and antibiotics, with specific suppressive activity against soilborne pathogens. Antagonistic pseudomonads, including *Pseudomonas chlororaphis*, play a role in white root rot suppressiveness (Cazorla *et al.*, 2006; Calderón *et al.*, 2014). However, other types of rhizobacterial taxa may differ in prevalence between suppressive and conducive soils, suggesting that the microbial

basis of white root rot could be far more complex than solely a *Pseudomonas* property; it has also been observed for other pathosystems such as *Thielaviospsis basicola*-mediated black root rot of tobacco (Almario *et al.*, 2014).

5. Conclusion

In conclusion, and taking together the results obtained in this work and in previous works related, a theoretical model about the role of the microorganisms in enhancing suppressiveness after amendment with composted almond shells can be proposed (Figure 15). Soil amendments with composted almond shells resulted in an extra input of organic matter rich in lignin that could be initially degraded by fungal members of the community (such as *Dothideomycetes*) and *Actinobacterias*. Lignin degradation from composting almond shells would produce a progressive release to the soil of more simple compounds.Those compounds, together with others also present in the almond shells, could lead to an increase in carbon sources available, such as cellulose, hemicellulose, and aromatic compounds. At this point, some *Proteobacteria* already present in the soil (such as *Gammaproteobacteria* and *Betaproteobacteria*) could take advantage metabolizing that available organic matter, thus slightly enhancing their population. These groups of microorganisms could harbor, among other, genes involved in antifungal enzymatic activities and production of antimicrobial compounds that could have an effect on the interaction with other microbes. The resulting modified microbiota after addition of composted almond shells could be more active against some groups of phytopathogenic fungi (as *Xylariales*, where *R. necatrix* is included) finally showing a phenotype of induced suppressiveness effect.

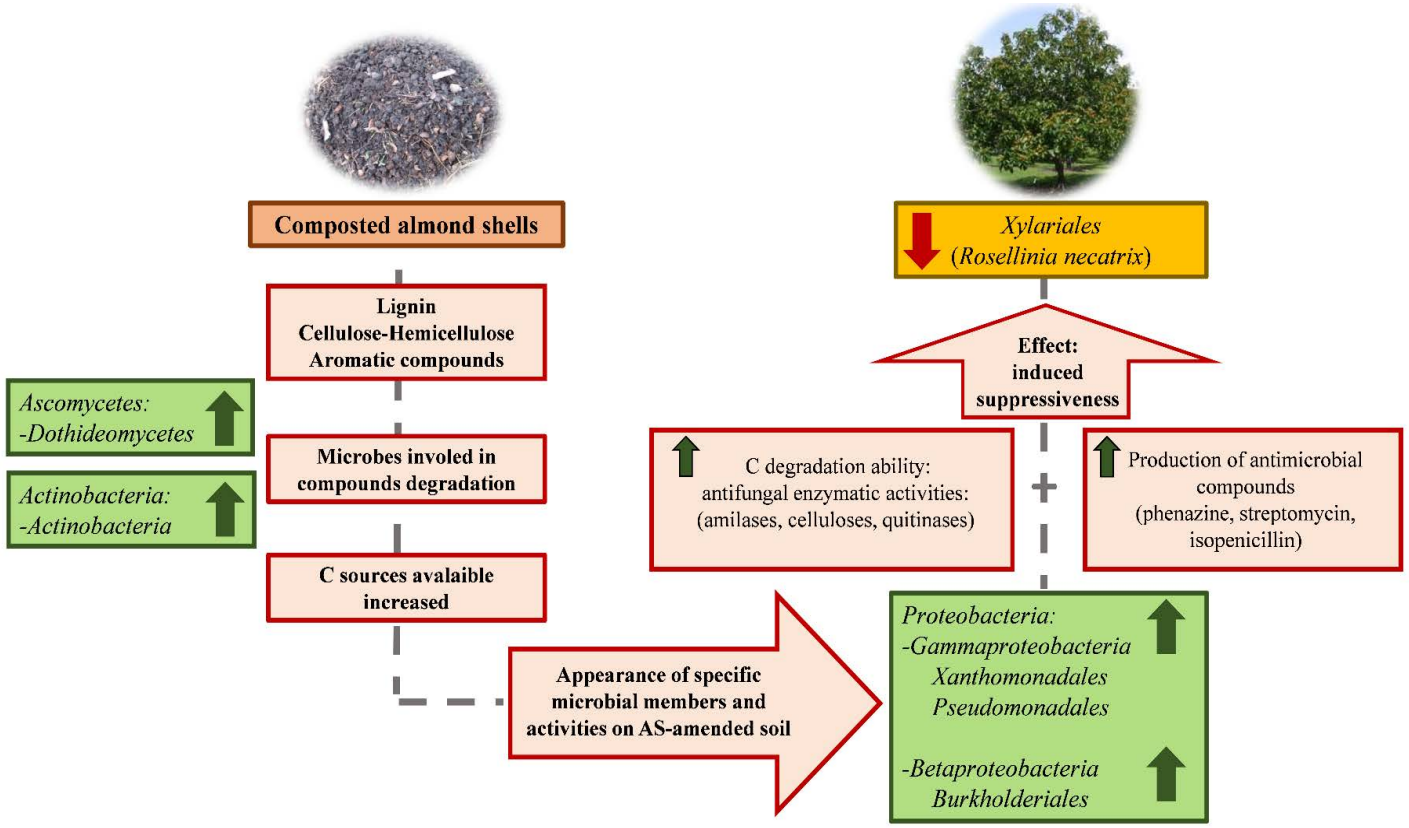


Figure 15: Hipothetical mode of action of almond shells amendment. Theoretical model proposed about the role of microorganisms in increasing suppressiveness after adition of composted almond shells to agricultural soil. The extra input of organic matter rich in lignin could be degraded by *Ascomycetes* and and *Actinobacterias*. An increase in carbon sources available occur and other polysacharides, as cellulose and hemicellulose, and aromatic compounds could be metabolize by *Proteobacteria*. These groups of microorganisms produce enzymes with antifungal activities (such as chitinases) and antimicrobial compounds (such as phenazine). Modified microbiota could have specific activity against fungi pathogens as *Xilariales*, where *Rosellinia necatrix* is included. The effect of microbial community changes induced a suppressiveness in the agricultural soil.

CHAPTER III

Characterization of biocontrol strains after an amendment with composted almond shells from a suppressiveness-induced soil

SUBMITTED IN:

Vida, C., Cazorla, F.M. and de Vicente, A. (2017) Characterization of biocontrol strains after an amendment with composted almond shells from a suppressiveness-induced soil. *Res. Microbiol.*

Abstract

The improvement of soil quality in avocado crops through organic amendments with composted almond shells has a positive effect on crop yield and plant health and serves as an enhancement of soil suppressiveness against the phytopathogenic fungus *Rosellinia necatrix*. In previous studies, induced soil suppressiveness against this pathogen was related to the stimulation of *Gammaproteobacteria*, especially some members of *Pseudomonas* spp. with biocontrol-related activities. In this work, we isolated bacteria from this suppressiveness-induced amended soil using a selective medium for *Pseudomonas*-like microorganisms. We characterized the obtained bacterial collection to aid in identification, including metabolic profiles, antagonistic responses, hybridization to biosynthetic genes of antifungal compounds, production of lytic exoenzymatic activities, and plant growth promotion-related traits, and sequenced and compared amplified 16S rDNA genes from some representative bacteria. The final selection of representative strains mainly belonged to the genus *Pseudomonas* but also included the genera *Serratia* and *Stenotrophomonas*. Their biocontrol-related activities were assayed using the experimental avocado model, and the results showed that all the selected strains protected the avocado roots against *R. necatrix*. This work confirmed the biocontrol activity of these *Gammaproteobacteria*-related members against *R. necatrix* following specific stimulation in a suppressiveness-induced soil after a composted almond shell application.

1. Introduction

Southern Spain is one of the most relevant zones in the Mediterranean area for the avocado crop (*Persea americana* Mill.) and is the main European producer (Robledo and Hermoso, 2009). In this area, one of the main phytopathological problems of the avocado crop is white root rot (WRR) caused by the fungus *Rosellinia necatrix*, which is considered an emergent pathogen (Pliego *et al.*, 2012). Over the past several decades, integrated management strategies to control white root rot have been attempted, including physical, chemical and biological control approaches (López-Herrera *et al.*, 1998; López-Herrera and Zea-Bonilla, 2007; González-Sánchez *et al.*, 2013). For biological control, several examples of microorganisms with biocontrol abilities against *R. necatrix* have been reported using different strategies, including antagonism (Cazorla *et al.*, 2006, 2007), competition for niches and nutrients (Pliego *et al.*, 2008), or induction of systemic resistance and/or predation (Ruano-Rosa *et al.*, 2014). However, additional management types can increase the soil health and yield in avocado crops. These actions included the use of organic amendments or mulches (Moore-Gordon *et al.*, 1997; Wolstenholme *et al.*, 1997; Cretoiu *et al.*, 2013). In a recent study, the application of composted almond shells as a mulch for avocado trees was considered an useful approach with an effect against white root rot through the induction of suppressiveness (Bonilla *et al.*, 2015).

Suppressiveness-induced soils after the application of composted almond shells have been reported to enhance some microbial representatives, many of them from *Gammaproteobacteria*; several members could be involved in the suppressive phenotype (Vida *et al.*, 2016). Isolates belonging to the *Gammaproteobacteria* class are subjects of special interest for biocontrol, and some biocontrol-related species of the genus *Pseudomonas* spp. have been extensively described (Weller *et al.*, 2002). Moreover, other soil members of this class, such as *Stenotrophomonas* spp. and *Serratia* spp., have also received interest due to their plant growth-promoting and plant

protection abilities (Berg, 2000; Alavi *et al.*, 2013; Beneduzi *et al.*, 2013;). Therefore, class *Gammaproteobacteria* (especially members overrepresented after amendment with composted almond shells) could be considered a good source for the isolation of biocontrol agents with diverse modes of action against the soilborne phytopathogen *R. necatrix*.

In this work, we report the isolation of *Gammaproteobacteria* from soil samples of avocado trees under ecological management of amendments with composted almond shells. The obtained collection of bacterial isolates was preliminary grouped using general tests. Moreover, additional characterization of biocontrol-related traits was completed, including *in vitro* antagonistic activity assays against soilborne phytopathogenic fungi, the detection of antimicrobial biosynthetic genes or the production of lytic exoenzymes. Selected representatives from different groups were analysed for plant growth promotion activities, and their biocontrol abilities were confirmed using the avocado/*R. necatrix* test system.

2. Materials and methods

2.1 Soil sampling

Soil samples were obtained from a 2.5-km^2 experimental field located at the Experimental Station 'La Mayora' [IHSM-UMA-CSIC, Málaga, Spain (36°75'N, 4°04'O)] in Malaga Province. A selection of 5 pairs of trees under ecological management (massive application of composted almond shells in 2002, 2007 and 2012) randomly placed throughout the avocado orchard were sampled (Figure 16). Field soil samples allocated underneath avocado trees amended with composted almond shells (AS) were taken to perform the bacterial isolation. To obtain a composite soil sample, we selected two sampling distal points 1.5 m around the trunk

base for each pair of trees (4 samples per pair of trees). The upper layer of compost was carefully removed, and approximately 0.5 kg of soil (100-150 g per sampling point) was collected at a 15-cm depth and merged to obtain a composite soil sample per pair of trees. The soil samples were placed in cold storage and transported to the laboratory, where they were sieved through a 20-mm mesh and immediately used.

Figure 16: Experimental avocado field (orange line) located in Experimental Station 'La Mayora'. Pair of trees were under conventional and organic management. Those amended with composted almond shells have been used in this study (boxed in green).

2.2 Bacterial isolation

For each sampling point, 2 g of sieved soil was suspended in 20 ml of sterile saline solution with 1 g of sterile gravel (3-mm diameter) and mixed at 200 rpm for 30 min on an orbital shaker. Ten-fold serial dilutions were plated on previously described Pseudomonads selective medium composed of King's B (KB) agar with 75 mg of penicillin G, 45 mg of novobiocin and 100 mg of cycloheximide per litre (Larkin and Honeycutt, 2006). This medium allowed the isolation of both Pseudomonads and different Gram-negative bacteria with different metabolic profiles (Sands and Rovira, 1970). The plates were incubated at 25°C for 48 h. A collection of isolates (n= 246) was constructed by selecting representatives from the most abundant colonies among

colonies with different morphologies (Figure 17). The bacterial collection of soil isolates was stored at -80°C. The isolates were characterized using the different approaches described below with the reference strains listed in Table 5.

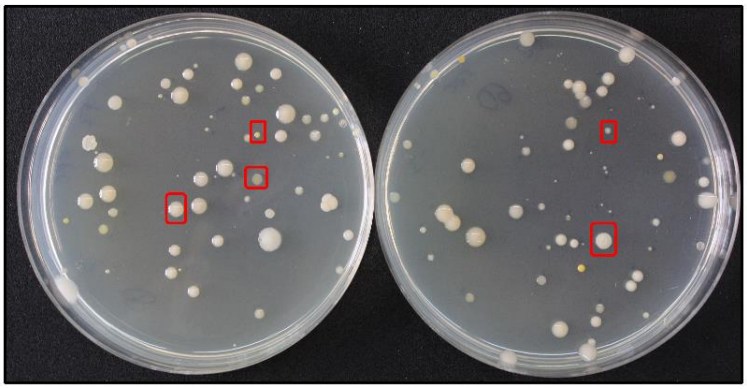


Figure 17: Different colony morphology (boxed in red) observed in KB medium supplemented with appropriate antibiotics for isolation of Pseudomonads and related groups.

2.3 Preliminary metabolic characterization

General basic metabolic characteristic tests were performed for the collection of isolates (n=246). The cell wall type was determined using a nonstaining method (KOH) (Buck, 1982). The catalase and oxidase reactions, oxidative/fermentative metabolism of glucose and production of fluorescent pigments were tested using standard procedures (Brenner *et al.*, 2005).

Table 5: Characteristics of bacterial control strains used in this study.

Strains (Reference)	Characteristics	Antagonism			Enzymatic activities						Plant growth promotion			Antimicrobial biosynthetic genes					
		Rn	*Fo*	*Pc*	Lip	Pro	Ami	Glu	Cel	Chi	Pho	Sid	SGP	HPR	PCA	HCN	PLT	PRN	DAPG
Bacillus amyloliquefaciens CECT 8237 (Magno et al.,)	Wild type, isolated from Spanish healthy leaf areas of cucurbit plants affected by powdery mildew	■	■	■	□	■	□	■	■	□	□	□	■	□	□	□	□	□	□
Pseudomonas chlororaphis PCL1391 (Chin-A-Woeng et al., 1998)	Wild type, isolated from Spanish tomato rhizosphere	■	■	■	■	■	□	□	□	■	□	■	□	□	■	□	□	□	□
Pseudomonas chlororaphis PCL1601 (Cazorla et al., 2006)	Wild type, isolated from Spanish avocado rhizosphere	■	□	■	■	■	□	□	□	□	□	■	□	□	■	■	□	□	□
Pseudomonas chlororaphis PCL1606 (Cazorla et al., 2006)	Wild type, isolated from Spanish avocado rhizosphere	■	■	■	■	■	□	□	□	□	□	■	□	■	□	■	□	■	□
Pseudomonas fluorescens BL915 (Hill et al., 1994)	Wild type, isolated from plants growing in blackland soil in Hill County, Tex	■	■	■	□	■	□	□	□	□	□	■	□	■	□	■	□	■	□
Pseudomonas fluorescens WCS365 (Geels and Schippers 1983)	Wild type, isolated from potato roots in Baarn, The Netherlands	□	□	■	□	□	□	□	□	□	□	■	□	□	□	□	□	□	□
Pseudomonas protegens Pf5 (Howell and Stipanovic, 1979)	Wild type, isolated from rhizosphere soil of cotton seedlings in National Cotton Pathology Research Laboratory, Tex	■	■	■	■	■	■	□	□	■	■	■	□	□	□	■	■	■	■
Pseudomonas pseudoalcaligenes AVO110 (Pliego et al., 2007)	Wild type, isolated from Spanish avocado rhizosphere	■	□	□	□	□	□	□	■	□	□	□	□	□	□	□	□	□	□

Black box: positive result; white box: negative result; R.n.: *Rosellinia necatrix* CH53; F.o.: *Fusarium oxysporum* sp *radicis-lycopersici* ZUM2407; P.c.: *Phytophthora cinnamomi* 344; Lip: lipase; Pro: protease; Ami: amilase; Glu: B-glucanase; Cel: cellulase; Chi: chitinase; Pho: phosphate solubilization; Sid: siderophores; SGP: seed growth promotion; HPR: 2-hexyl 5-propyl resorcinol; PCA: phenazine-1-carboxylic acid; HCN: hydrogen cyanide; PLT: pyoluteorin; PRN: pyrrolnitrin; DAPG: 2,4-diacetylphloroglucinol..

2.4 Antagonistic activity

The antagonistic ability of the 246 bacterial isolates was studied at 25°C on potato dextrose agar (PDA, Difco Laboratories, Detroit, USA) using a dual plate assay as previously described (Cazorla *et al.*, 2006). The soilborne phytopathogenic fungal strains *Rosellinia necatrix* CH53 (López-Herrera and Zea-Bonilla, 2007) and *Fusarium oxysporum* sp. *radicis-lycopersici* ZUM2407 (Cazorla *et al.*, 2006) and the phytopathogenic oomycete *Phytophthora cinammommi* 344 strain (Pérez-Jiménez, 2008), were used in this study. Bacterial isolates inhibiting mycelial growth after 5 days at 25°C in the dark were reported as antagonistic.

The presence of biosynthetic genes responsible for the production of well-known antifungal compounds mainly produced by *Pseudomonas* spp. but also produced by other Gram-negative bacteria (Raaijmakers *et al.*, 2002) were analysed by colony blotting following the previously described procedure (Matas *et al.*, 2014). For this purpose, the antifungal biosynthetic genes phenazine-1-carboxylic acid (PCA), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT), 2-hexyl 5-propyl resorcinol (HPR) and hydrogen cyanide (HCN) were detected using colony blotting assays. DNA probes were obtained by PCR amplification using specific primer pairs (Table 6) from the reference biocontrol strains *Pseudomonas chlororaphis* PCL1606 (for HPR, HCN, and PRN), *Pseudomonas chlororaphis* PCL1391 (for PCA) and *Pseudomonas protegens* Pf5 (for DAPG and PLT) (Castric, 1975; Howell and Stipanovic, 1979; Chin-A-Woeng *et al.*, 1998; Cazorla *et al.*, 2006). The DNA probes obtained from the purified PCR amplicons were labelled with digoxigenin (DIG) using the DIG-High Prime labelling kit (Roche, Basel, Switzerland) following the manufacturer's instructions. The probe sequences were submitted to the National Center for Biotechnology Information (NCBI) under accession number ID1917757.

Bacterial colonies from the culture plates were spotted onto a nylon membrane (Nytran®N, GE Healthcare Life Science, USA) and processed by colony blotting as previously described (Matas *et al.*, 2014). The spotted membrane was dried, and the DNA was crosslinked in a UV chamber. Prior to DNA fixation, 0.5 µl of DNA was extracted from the control reference strains using the UltraClean® Microbial DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions and was then added to the test membrane.

For membrane hybridization, we used the DIG Easy Hyb kit (Roche, Basel, Switzerland) following the manufacturer's instructions. Immunological detection was performed with an anti-digoxigenin antibody conjugated to alkaline phosphatase. The DIG-labelled nucleic acids were detected by chemiluminescence using a Molecular Imager ChemiDoc system (Bio-Rad).

Table 6: Oligonucleotide sequences used in this study to obtain probes for detection of genes responsible of antifungal production.

Antibiotic	Biosynthetic gene	Primer name	Primer sequence 5'-3'
Hydrogen cyanide (HCN)	*hcn*BC	hcnBC F	TGCGGCATGGGCGTGTGCCATTGCTGCCTGG
		hcnBC R	CCGCTCTTGATCTGCAATGCAGGCC
Phenazine-1-carboxylic acid (PCA)	*phz*R	phzR F	TTCATTCGTCGGCTGAG
		phzR R	ATCCTCGCCATCAAACTC
2-hexyl 5-propyl resorcinol (HPR)	*dar*B *	darB F	CCTGCCAGACCACTTAG
		darB R	GAAGGTGGTCGATCCAGTCG
Pyoluteorine (PLT)	*plt*A	pltA R	CGAGAAAGCCAACTTCCC
		pltA F	TTCAGGCGGTAATAGAGCG
Pyrrolnitrin (PRN)	*prn*A	prnA F	GGACCTGTTCATCGACTGCT
		prnA R	AAGGTGCGTTCCACTACAGG
2,4-diacetylphloroglucinol (DAPG)	*phl*D	phlD F	ACCCACCGCAGCATCGTTTAT
		phlD R	CCGCCGGTATGGAAGATGAAA

*Published in Calderon *et al.*, 2013

2.5 Enzymatic activities

Plate-based assays were used for the direct characterization of bacterial hydrolytic activities. We tested for lipase, protease, amylase, β-glucanase, cellulase, and chitinase enzymatic activity. Luria Bertani (LB) agar (0.8%) plates were supplemented with different test substrates to evaluate each enzymatic activity after incubation at 25°C for 2-5 days. LB plates with Tween 80 (2%, Sigma-Aldrich, Madrid, Spain) were used for lipase detection (Howe and Ward, 1976); the assay was considered positive when the formation of a calcium oleate precipitate was observed. LB plates with powdered milk (3%) were used for protease detection (Gerhardt, 1994) and plates with colloidal chitin (0.2%, Sigma-Aldrich, Madrid, Spain) were used for chitinase detection (Murthy and Bleakley, 2012); in the protease and chitinase assays, the presence of clearing zones (halos) surrounding bacterial growth was considered a positive result. LB plates with starch (0.75%, Panreac, Barcelona, Spain) were used to evaluate amylase activity, whereas plates with lichenan (0.1%, Megazyme, Barcelona, Spain) were used to detect β-glucanase activity (Walsh *et al.*, 1995) and plates with 1-carboxymethylcellulose (0.75%, Panreac, Barcelona, Spain) were used to detect cellulase activity (Hankin and Anagnostakis, 1995); for the amylase, β-glucanase and cellulase assays, the plates were stained with Congo red (0.3%) for 30 min, and clearing halos around the colonies represented a positive enzymatic response.

2.6 PGP-related activities

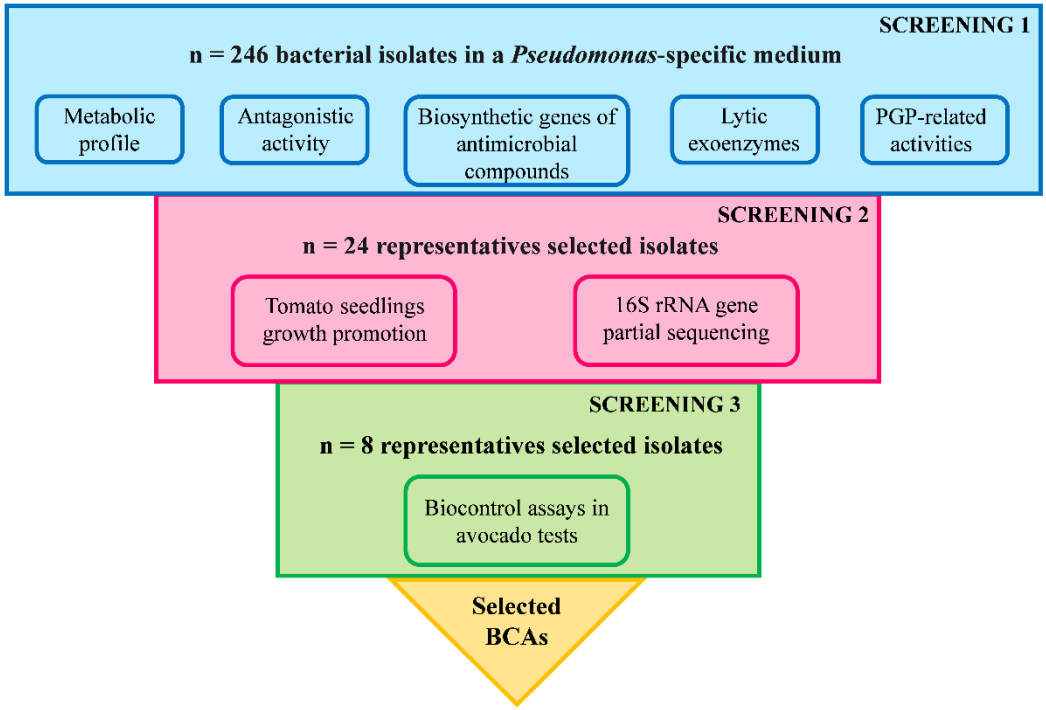
The collection of 246 bacterial isolates was tested for phosphate solubilisation and siderophore production as activities related to plant growth promotion (PGP). To identify phosphate-solubilizing activity, bacterial isolates were grown in glucose-yeast (GY) broth containing 8 g of agar, 50 ml of K_2HPO_4 (10%) and 100 ml of $CaCl_2$ (10%) per litre. The plates were incubated for 5 days at 25°C, and the formation of visible

clearing halos around the tested colonies indicated phosphate solubilisation (Sylvester-Bradley *et al.*, 1982).

Siderophore-producing bacteria were detected using a mixture of M9 medium salt (1x) without Na_2HPO_4, 30.24 g of PIPES buffer, 9 g of agar, 30 ml of casamino acid solution (10%), 10 ml of glucose solution (20%) (Cordero *et al.*, 2012) and 100 ml of chromazurol S complex [CAS/iron(III)/hexadecyl-trimethyl ammonium bromide] per litre. The plates were incubated at 25°C for 48 h. Siderophore-producing bacteria formed a yellow-orange halo in the blue-green medium background.

From the isolate collection, 24 representatives were selected based on their differential responses in the previous tests (Figure 18). *In vitro* seedling growth promotion assays were performed following the procedure of Ryu *et al.*, 2005 with modifications. The 24 representative selected isolates were assayed for the ability to promote tomato (*Solanum lycopersicum* L.) seed germination and plant growth. Tomato seeds (c.v. "Moneymaker") were disinfected by immersion in 0.1% NaOCl for 20 min and then washed (20 min) with sterile distilled water. Ten tomato seedlings were dipped in 1 ml of a 1×10^9 cfu/ml suspension of each selected test isolate. *Bacillus amyloliquefaciens* CECT8237 was used as a positive control for PGPR activity (Magno *et al.*, 2015) and *P. chlororaphis* PCL1606 was used as a negative control. After 20 min of inoculation, the excess suspension was poured off, and the inoculated seedlings were transferred to test tubes with 3 ml of Murashige and Skoog agar (0.8%) and incubated at 25°C in the dark for 3 days. Then, the seedlings were grown at 25°C under a diurnal cycle of white light/dark (16/8 h). The average fresh weight (mg) was calculated after 10 days of growth.

Figure 18: Workflow used in this study to characterize soil bacterial isolates with biocontrol ability against avocado fungal pathogen *Rosellinia necatrix*.



2.7 Identification using 16S rRNA gene partial sequencing

To help characterize the 24 selected isolates, sequencing of the variable region of the bacterial 16S rRNA gene was performed. PCR amplification was performed using the primers 8F (5′-AGR GTT YGA TYM TGG CTC AG -3′) and 1391R (5′-GAC GGG CGG TGT GTR CA-3′) (Klindworth *et al.*, 2013). The PCR products were checked by electrophoresis in an agarose gel (0.8%) and sequenced by Macrogen Europe (Amsterdam, the Netherlands). Identity studies comparing the partial sequences to previously deposited sequences were performed using the National Center for Biotechnology Information (NCBI) GenBank Blast software (Bethesda, MD, USA).

The corresponding sequences displaying a similarity higher than 85% were deposited in the GenBank database (Table 7).

Table 7: Information of partial sequences of 16S rRNA gene obtained by PCR methology from 24 selected bacterial isolates submitted to GenBank database.

Selection code	Accession number	Sequence length *	16S rDNA sequence **	
			Identity to:	%
1	KY079264	395	*Pseudomonas* sp.	97
2	KY079265	1024	*Serratia* sp.	95
3	KY079266	750	*Pseudomonas sp.*	98
4	KY079267	1000	*Pseudomonas sp.*	96
5	KY079268	800	*Pseudomonas sp.*	96
6	KY079269	850	*Stenotrophomonas sp.*	99
7	KY079270	700	*Serratia sp.*	94
8	-	-	Unidentified	-
9	KY079271	450	*Serratia sp.*	96
10	-	-	Unidentified	-
11	-	-	Unidentified	-
12	-	-	Unidentified	-
13	KY079272	450	*Serratia sp.*	91
14	KY079273	800	*Pseudomonas sp.*	99
15	KY079274	1050	*Pseudomonas sp.*	99
16	KY079275	850	*Pseudomonas sp.*	85
17	KY079276	950	*Enterobacter sp.*	85
18	KY079277	600	*Serratia sp.*	92
19	KY079278	800	*Stenotrophomonas sp.*	99
20	KY079279	800	*Pseudomonas sp.*	95
21	KY079280	1050	*Stenotrophomonas sp.*	97
22	-	-	Unidentified	-
23	-	-	Unidentified	-
24	-	-	Unidentified	-

*, pair of bases, **, percentage of identity with others bacterial uploaded sequences in GenBank database obtained by Blast tool.

2.8 Biocontrol activities

Based on the previous results, 8 representative bacterial isolates were selected for the biocontrol experiments (Figure 18). Biocontrol assays against white root rot caused by

the virulent strain *R. necatrix* CH53 were conducted using the susceptible pathosystem on avocado plants (Cazorla *et al.*, 2006). Briefly, the biocontrol assays were performed with six-month-old commercial avocado plants supplied by Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain). The roots from the avocado plants were disinfected by immersion in 0.1% NaOCl for 20 min and then washed (20 min) with sterile distilled water. The roots were bacterized by immersion in a suspension of the bacterial isolates or mixtures (10^8 cfu/ml) or in sterile LB medium as a negative control for 20 min. The avocado plants were placed into square plastic pots containing potting soil. Fungal infection with *R. necatrix* was performed using infected wheat grains as previously described (Freeman *et al.*, 1986). Non-bacterized plants were used as the controls. Fifteen avocado plants were tested per treatment. The plants were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms of the disease in the avocadoes were recorded on a scale of 0–3, and the disease index (DI) was calculated at the end of the assay (Cazorla *et al.*, 2006).

2.9 Statistical methods

The biocontrol and seed growth promotion test data were statistically analysed using analysis of variance (Sokal and Rohl, 1986), followed by Fisher's least significant difference test ($p = 0.05$) using the SPSS 22 software (SPSS Inc., Chicago).

3. Results

3.1 Isolation and characterization

A collection of culturable bacteria from composted almond shells-amended soil was constructed using a selective medium for Pseudomonads (Larkin and Honeycutt, 2006) and related groups (Sands and Rovira, 1970). A total of 246 bacterial isolates were initially selected based on the abundance and differences in colony morphology displayed on the plates. Physiological and metabolic tests were performed to group the isolates. All 246 isolates responded as Gram-negative and catalase-positive bacteria. The diversity observed in the additional tests allowed the bacterial isolates to be clustered into 5 groups. One hundred and forty-eight isolates (60.2%) that were glucose fermentative and oxidase negative with non-fluorescent and non-pigmented colonies were grouped as *Enterobacteriaceae*-like (group A). Twenty-six isolates (10.5%) were glucose oxidative, oxidase positive and producers of fluorescent pigments under UV light; these isolates were considered fluorescent *Pseudomonadaceae*-like (group B). Eleven isolates (4.5%) were glucose oxidative, oxidase positive and non-producers of fluorescent pigment; thus, these isolates were grouped as non-fluorescent *Pseudomonadaceae*-like (group B'). Twelve isolates (4.9%) were glucose oxidative, oxidase negative and yellow pigmented colonies and were clustered as *Xanthomonadaceae*-like (group C). Finally, 49 bacterial isolates (19.9%) could not metabolize glucose and were oxidase negative and were grouped as unclassified isolates (group D).

3.2 Antagonistic activity of bacterial isolates from soil

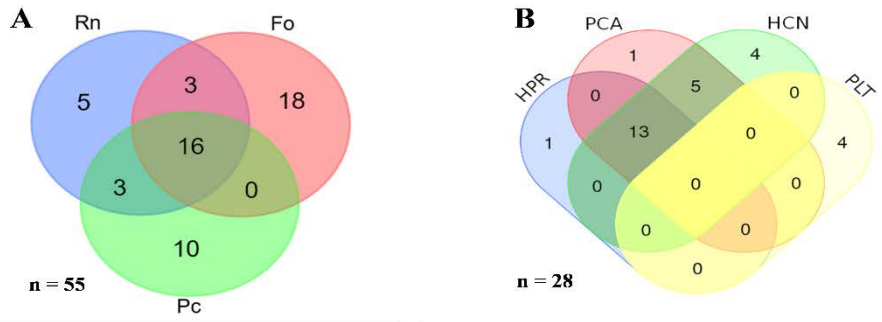
Further characterization of biocontrol-related traits was performed for the collection of 246 isolates. The antagonistic abilities against the phytopathogenic soilborne fungi *R. necatrix* CH53 (Rn) and *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407 (Fo) and

the phytopathogenic oomycete *P. cinnamomi* 344 (Pc) were assessed using dual plate assays. A total of 22% of the isolates exhibited antagonistic behaviour (n=55) (Figure 19A). The antagonistic isolates were mainly present in groups A (n=31) and B+B' (n=17+4), with many representative isolates displaying antagonism to all three soilborne pathogens assayed (A=8 and B=8). The number of strains antagonistic to the avocado phytopathogens *R. necatrix* and *P. cinnamomi* were very similar (n=27 and n=29 isolates, respectively). However, more strains were antagonistic to *F. oxysporum* (n=37).

To gain insights into the nature of the antagonistic activity, colony blotting was performed using digoxigenin-labelled probes to assess antifungal compounds (Castric, 1975; Howell and Stipanovic, 1979; Chin-A-Woeng *et al.*, 1998; Cazorla *et al.*, 2006). A total of 11% of the bacterial isolates exhibited hybridization to biosynthetic antimicrobial product genes (n=27). Group B+B' showed the highest number (n=12+4) of representatives with hybridization to the different probes (Figure 19B). Some of the bacterial isolates showed multiple hybridization signals to 2 or 3 antimicrobial biosynthetic genes. Specifically, 13 isolates showed the presence of both HPR biosynthetic genes and PCA and HCN biosynthetic genes; this group was the most abundant. In this specific group, we primarily found 8 fluorescent *Pseudomonadaceae*-like isolates as the more represented group of strains (group B) as well as 3 hypothetical *Enterobacteriaceae*-like (group A) bacteria. No hybridization signals were observed for the PRN and DAPG biosynthetic genes with the exception of the control strains PCL1606, BL915 and Pf5 (Table 8).

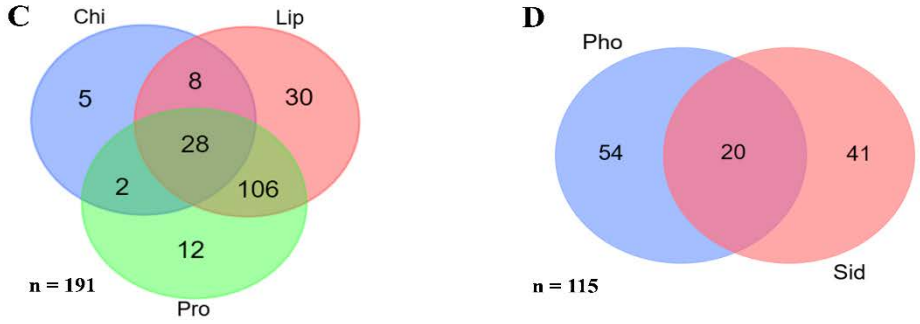
3.3 Enzyme production

The total collection of 246 bacterial isolates were analysed for the production of exoenzymatic activities (lipases, proteases, amylases, β-glucanases, cellulases and chitinases). Seventy-eight percent of the isolates (Figure 19C) were positive for at least



Antagonism to:							
	Rn	Fo	Pc	Rn Fo	Rn Pc	Rn Fo Pc	Σ
A	4	13	3	3		8	31
B		3	6			8	17
B'		1			3		4
C			1				1
D	1	1					2

Presence of biosynthetic genes of:							
	HPR	PCA	HCN	PLT	PCA HCN	PCA HCN HPR	Σ
A			1	3	2	3	9
B	1		3			8	12
B'		1		1	3		4
C						1	1
D						1	1



Exoenzymatic activities								
	Chi	Lip	Pro	Chi Lip	Chi Pro	Lip Pro	Chi Lip Pro	Σ
A	4	27	2	8	1	48	20	110
B	1		7		1	8		17
B'		1				3	2	6
C		2	1			7	1	11
D			2			40	5	47

PGP-related activities				
	Pho	Sid	Pho Sid	Σ
A	44	14	18	76
B	4	7	1	12
B'	1	1		2
C		2		2
D	5	17	1	23

Groups: **A** = *Enterobacteriaceae*-like group; **B** = fluorescent *Pseudomonadaceae*-like group; **B'** = no fluorescent *Pseudomonadaceae*-like group; **C** = *Xanthomonadaceae*-like group; **D** = Unclassified isolates

Figure 19: Characterization of biocontrol- and PGP-related traits for the collection of bacterial isolates (n= 246) and the number of isolates displaying each response (n). **A)** Antagonism against soilborne phytopathogens. (**B)** Detection of biosynthetic genes for antifungal production. (**C)** Enzymatic activities. (**D)** PGP-related activities.
Rn: *Rosellinia necatrix*; Fo: *Fusarium oxysporum* f. sp. *radicis-lycopersici*; Pc: *Phytophthora cinnamomi*; Lip: lipase; Pro: protease; Chi: chitinase; Pho: phosphate solubilisation; Sid: siderophores; HPR: 2-hexyl 5-propyl resorcinol; PCA: phenazine-1-carboxylic acid; HCN: hydrogen cyanide; PLT: pyoluteorin; PRN: pyrrolnitrin; DAPG: 2,4-diacetylphloroglucinol.

one of the enzymatic activities tested (n=191). Of the bacterial isolates with enzymatic degradation abilities, 172 isolates exhibited lipase activity, 148 strains produced proteases and 43 isolates had positive results for chitinase. Some of the isolates shared the production of 2 or 3 exoenzymes. Forty-three percent (n=106) of the bacteria exhibited lipase and protease activity (A=48, B+B'=8+3, C=7, and D=40). Furthermore, 28 strains produced lipase, protease and chitinase enzymes (A=20, B'=2, C=1, and D=5). Eight strains shared lipase and chitinase production, all of which were from the *Enterobacteriaceae*-like group, and only 2 of the isolates produced the protease and chitinase enzymes. No amylase, β-glucanase or cellulase activity was detected in any of the isolates analysed in this study.

3.4 Plant growth promotion-related activities

Plant growth promotion-related activities were analysed for the bacterial isolate collection (n=246). Seventy-four percent of the isolates only had the capacity to solubilize a non-soluble phosphate source (A=44, B+B'=4+1, and D=5), and only 61 isolates produced siderophores (A=14, B+B'=7+1, C=2, and D=17) (Figure 19D). Twenty isolates produced both activities, 18 of which were *Enterobacteriaceae*-like.

To gain insights into the effective contribution of representative selected isolates (n=24) to plant growth, a tomato seedling growth promotion assay was performed. The bacterial isolates '17' (*Enterobacteriaceae*-like) and '20' (fluorescent *Pseudomonadaceae*-like, Figure 20) showed a significant increase in the average fresh weight (mg) at the end of the experiment similar to the positive control plant growth-promoting bacterium *Bacillus amyloliquefaciens* CECT8237 (Magno *et al.*, 2015).

In contrast, inoculation with strains '4' and '8' caused a significant decrease in the average fresh weight similar to the results obtained with *Pseudomonas chlororaphis* PCL1606, which was a previously described non-PGPR control strain (Tienda *et al.*, 2016). The remainder of the selected bacteria did not show significant differences compared with the germination control treatment (seeds inoculated only with LB medium).

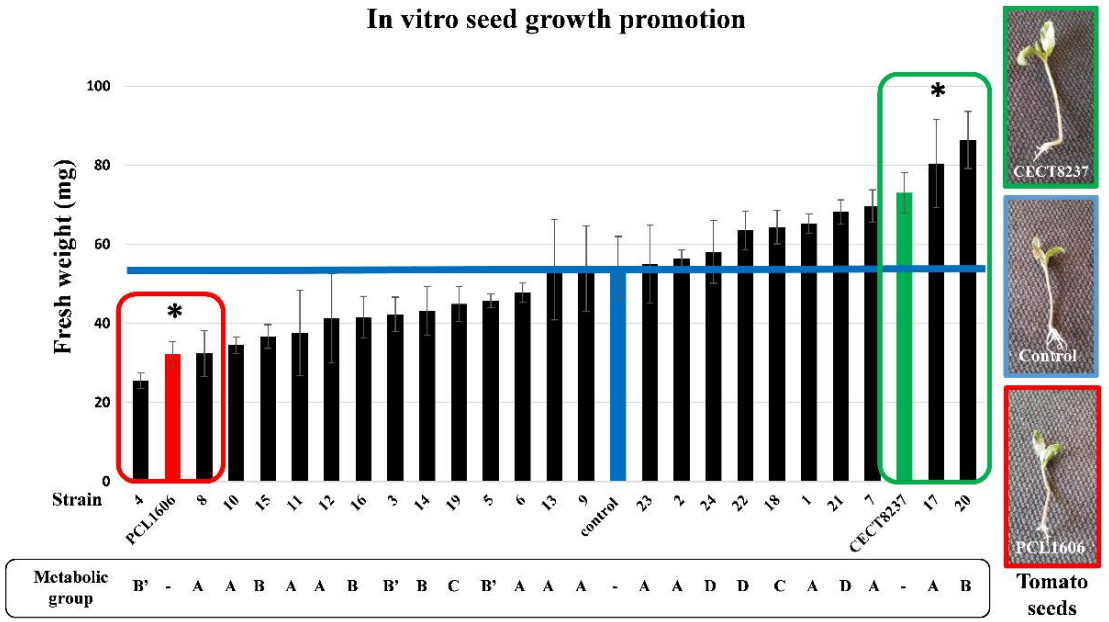


Figure 20: Tomato seed growth promotion assay using selected isolates. Tomato seeds were inoculated with the strains prior to transfer to Murashige and Skoog agar and incubated at 25°C with 16:8 h of light:dark. The fresh weights of the plants were scored 10 days after bacterization. The data were analysed for significance using an arcsine square root transformation with analysis of variance followed by Fisher's least significant difference test (p<0.05). Values with asterisks denote a significant difference with respect to the control strain.

3.5 16S rRNA gene partial sequencing analysis

An identity analysis of partial 16S rRNA gene sequences was performed for representative selected isolates (n=24). The 16S rDNA sequences of the identified strains were submitted to the GenBank database under accession numbers KY079264-KY079280. Seven partial 16S rRNA gene sequences corresponding to the strains "8", "10", "11", "12", "22", "23" and "24" (Table 7) were not deposited due to a similarity lower than 85% to sequences previously uploaded in the NCBI database and were considered unidentified strains in this work (Table 8).

Hypothetically, all of the identified selected isolates belonged to the group of *Gammaproteobacteria*, which conformed to all of the *Pseudomonas* spp. characteristics, as well as a member each from *Serratia* spp. and *Stenotrophomonas* spp. Only 12 of the isolates could be identified with a percent identity higher than 95% (Table 7), as belonging to different metabolic profile groups. However, a putative genus name was only assigned for 9 of the bacterial isolates with compatible 16S rRNA partial sequences and metabolic group results (A=2, *Serratia sp*.; B+B'=6, *Pseudomonas sp*.; and C=1, *Stenotrophomonas sp.*; Table 8). For the remainder of the selected isolates (n=8), we could not provide a hypothetical genus name due to incompatibility between the 16S rRNA partial sequences and the metabolic group results; these strains were considered *incertae sedis* strains in this work (Table 8).

Table 8: Characterization of selected isolates from almond shells amended soil.

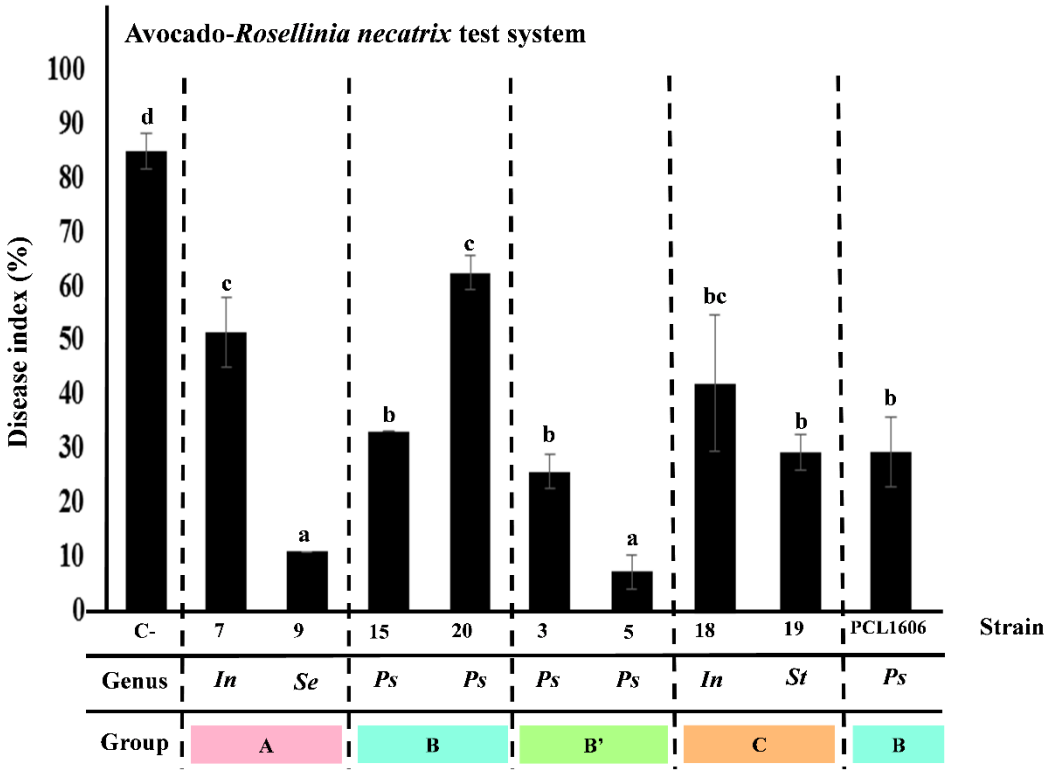
Selection code	Accession number	16S rDNA sequence	Metabolic pattern	Antagonism			Detectios of antimicrobial biosynthetic genes				Enzymatic activities			Plant growth promotion			Biocontrol activitiy against *R.n*
		Hypothetical genus	Group	*R.n.*	*F.o.*	*P.c.*	HPR	PCA	HCN	PLT	Lip	Pro	Chi	Pho	Sid	SGP	Avocado
1	KY079264	*Incertae sedis*	A	■	□	□	□	□	□	■	■	■	□	■	□	□	■
2	KY079265	*Serratia* sp.	A	■	■	□	□	□	□	■	■	■	□	□	□	□	■
6	KY079269	*Incertae sedis*	A	■	□	□	□	□	□	□	■	□	■	■	■	□	■
7	KY079270	*Incertae sedis*	A	■	■	■	□	□	□	□	■	■	■	□	■	□	■
8	-	Unidentified	A	□	□	□	□	■	■	□	■	□	□	■	□	□	■
9	KY079271	*Serratia sp.*	A	■	■	■	□	□	□	□	■	□	□	□	□	□	■
10	-	Unidentified	A	■	■	■	□	■	■	□	■	■	□	□	□	□	■
11	-	Unidentified	A	■	■	■	□	□	□	□	■	□	□	□	□	□	■
12	-	Unidentified	A	□	□	□	□	□	■	□	■	■	□	■	□	□	■
13	KY079272	*Incertae sedis*	A	■	□	□	□	□	□	□	■	■	□	□	■	□	■
17	KY079276	*Incertae sedis*	A	□	■	□	□	■	■	□	□	□	□	■	■	■	■
23	-	Unidentified	A	□	□	■	□	□	□	□	■	■	■	■	□	□	■
14	KY079273	*Pseudomonas sp.*	B	■	■	■	■	■	■	□	■	■	□	□	□	□	■
15	KY079274	*Pseudomonas sp.*	B	■	■	■	■	■	■	□	■	■	□	□	□	□	■
16	KY079275	*Incertae sedis*	B	■	■	■	■	■	■	□	■	■	□	■	□	□	■
20	KY079279	*Pseudomonas sp.*	B	■	■	■	■	■	■	□	□	■	□	□	□	■	■
3	KY079266	*Pseudomonas sp.*	B'	■	□	■	□	■	■	□	■	■	□	□	□	□	■
4	KY079267	*Pseudomonas sp.*	B'	□	□	□	□	■	□	□	■	■	□	■	□	□	■
5	KY079268	*Pseudomonas sp.*	B'	■	□	■	□	■	■	□	■	■	■	□	■	□	■
18	KY079277	*Incertae sedis*	C	□	□	□	□	■	■	□	■	■	■	□	□	□	■
19	KY079278	*Stenotrophomonas sp.*	C	□	□	□	□	□	□	□	■	■	□	■	■	□	■
21	KY079280	*Incertae sedis*	D	□	□	□	□	□	□	□	■	■	■	□	■	□	■
22	-	Unidentified	D	□	□	□	□	□	□	□	■	■	■	■	□	□	■
24	-	Unidentified	D	□	□	□	□	■	■	□	■	■	□	□	□	□	■

Colorful box: positive result; white box: negative result; grey box: not tested; A: *Enterobacteriaceae*-like group; B: fluorescent *Pseudomonadaceae*-like group; B': non-fluorescent *Pseudomonadaceae*-like group; C: *Xanthomonadaceae*-like group; D: unclassified strains; R.n.: *Rosellinia necatrix*; F.o.: *Fusarium oxysporum*; P.c.: *Phytophthora cinnamomi*; HPR: 2-hexyl 5-propyl resorcinol; PCA: phenazine-1-carboxylic acid; HCN: hydrogen cyanide; PLT: pyoluteorin; Lip: lipase; Pro: protease; Chi: chitinase; Pho: phosphate solubilization; Sid: siderophores; SGP: seed growth promotion.

3.6 Biocontrol experiments

Based on the previous results and in an attempt to represent the maximum variability of the isolates (i.e., group, antagonist, and detection of antimicrobial biosynthetic genes), we selected 8 representative isolates to perform biocontrol assays using the avocado-*R. necatrix* test system. The selected isolates were '3', '5', '15', '20' (identified as *Pseudomonas spp*.), '9' (identified as *Serratia sp*.), '19' (identified as *Stenotrophomonas sp*.), '7' and '18' (identified as *incertae sedis* strains). At the end of the experiment (21 days for the avocado assay), we calculated the disease index for each treatment. The results were analysed with an ANOVA test. *P. chlororaphis* PCL1606 was included as a reference strain for biocontrol of avocado white root rot (Cazorla *et al.*, 2006). Non-bacterized plants were used as the negative control (Figure 21).

Remarkably, all of the isolates displayed significant biocontrol abilities to different extents against avocado white root rot. The *Pseudomonas* spp. strains tested showed very good protection ('5') to moderate protection ('20'), whereas the protection by the other strains was not significantly different ('3' and '15') compared with the positive control PCL1606. The same disease index displayed by the positive control strain PCL1606 was observed for the *Stenotrophomonas* sp. ('19'). However, one *Serratia* sp. ('9') showed an excellent performance in the avocado-*R-necatrix* test system. Finally, only the *incertae sedis* isolates '7' and '18' showed moderate although significant protection against *R. necatrix* (Figure 21).



In= *Incertae sedis*; ***Se***= *Serratia sp.*; ***Ps***= *Pseudomonas sp.*; ***St***= *Stenotrophomonas sp.*; **A**= *Enterobacteriaceae*-like group; **B**= fluorescent *Pseudomonadaceae*-like group; **B'**= no fluorescent *Pseudomonadaceae*-like group; **C**= *Xanthomonadaceae*-like group

Figure 21: Biocontrol of *Rosellinia necatrix*-induced white root rot in avocado plants resulting from the selected isolates. Roots from commercial avocado plants were inoculated prior to transfer to potting soil infested with *R. necatrix*. The plants were scored as sick or healthy 21 days after bacterization. The data were analysed for significance using an arcsine square root transformation with analysis of variance followed by Fisher's least significant difference test (p<0.05). Values with different letter denote significant differences. The error bars are the standard errors of three independent experiments.

4. Discussion

The roles of microbial communities in soil suppressiveness against a wide range of plant diseases have been described (Weller *et al.*, 2002; Mendes *et al.*, 2011; Bonilla *et al.*, 2015). In avocado crops, soil suppressiveness against the fungal pathogen *R. necatrix* can be induced by the application of composted almond shells, which also causes subtle changes in the bacterial community (Bonilla *et al.*, 2015; Vida *et al.*, 2016). Thus, specific members of *Gammaproteobacteria* show an increase in their relative abundance in this suppressiveness-induced soil, especially members of the *Pseudomonas* genus but also other bacteria such as *Serratia* or *Burkholderia* (Vida *et al.*, 2016). *Pseudomonas* sp. have been historically described as a source of biocontrol agents (BCAs) against different soilborne pathogens (Raaijmaker and Mazzola, 2012) including avocado phytopathogens (Cazorla *et al.*, 2006; Pliego *et al.*, 2008). Many species from this genus present a large set of weapons related to their biocontrol activity that can be used for their isolation, characterization and selection as BCAs. Thus, the isolation of specific biocontrol bacteria from induced-suppressive soil can be considered a good strategy to identify potentially beneficial bacteria.

After bacterial isolation using a specific medium with antibiotics (Larkin and Honeycutt, 2006), we obtained a collection of culturable bacteria from the suppressiveness-induced soil. To characterize these bacterial isolates, we used basic techniques for physiological and metabolic profile characterization, which helped group the isolates and facilitated their screening. In this study, general identification tests allowed the differentiation of 4 metabolic groups (*Enterobacteriaceae*-like group, fluorescent *Pseudomonadaceae*-like group, non-fluorescent *Pseudomonadaceae*-like group, and *Xanthomonadaceae*-like group) and a group of unclassified isolates with their own characteristic patterns. These results demonstrated that although the medium was initially developed to specifically isolate Pseudomonads from soils, the diversity

observed in the obtained isolates agreed with previous observations reporting specific isolation of Gram-negative bacteria with different metabolic profiles (Sands and Rovira, 1970).

In this study, we used a workflow of experiments chosen to characterize the collection of suppressiveness-inducing soil isolates (Lugtenberg and Kamilova, 2009). In this case, dual plate antagonism assays were performed against different fungal (*R. necatrix* CH53 and *F. oxysporum* f. sp. *radicis-lycopersici* ZUM2407) and oomycete (*P. cinnamomi* 344) phytopathogens. The results showed a slightly higher number of soil isolates with antagonistic activity to *F. oxysporum* (n= 37) than to the avocado pathogens *R. necatrix* (n= 27) and *P. cinnamomi* (n=29). Interestingly, many of the isolates with antagonism belonged to the *Enterobacteriaceae*-like group (A; n=31), followed by the fluorescent *Pseudomonadaceae*-like group (B; n=17) and the non-fluorescent *Pseudomonadaceae*-like group (B'; n=4). Several *Pseudomonas* spp. have been described previously as biocontrol agents for avocado crops (Cazorla *et al.*, 2006; Pliego *et al.*, 2008; González-Sánchez *et al.*, 2013). Previous studies showed that antagonism to different phytopathogens was a prevalent trait in the selected strains, suggesting that antagonism could be a useful strategy to select biocontrol strains for this plant-pathogen system (González-Sánchez *et al.*, 2013).

Fungal antagonism is usually mediated by different compounds, of which the antifungal antimicrobial compounds are of major importance (Raaijmakers *et al.*, 2002). A fast and easy-to-perform method to predict the putative production of antifungal compounds is genetic analysis of the presence of biosynthetic genes (Zhang *et al.*, 2006). The antifungal compounds produced by *Pseudomonas* spp. are well known and have been used in the colony blotting assays because these biosynthetic genes are available. Thus, the colony blotting detection assays for biosynthetic genes of antimicrobial compounds with antifungal activity showed a higher number of isolates with a triple combination of antibiotics (hydrogen cyanide (HCN), phenazine-

1-carboxylic acid (PCA) and 2-hexyl 5-propyl resorcinol (HPR)), mostly from the fluorescent *Pseudomonadaceae*-like group (B; n=8). Different examples of biocontrol fluorescent Pseudomonads have been described based on their ability to control a wide range of soilborne pathogens producing these mentioned antibiotics (Haas and Defago, 2005; Cazorla *et al.*, 2006). Nevertheless, we did not detect any isolates displaying hybridization signals to the biosynthetic genes for 2,4-diacetylphloroglucinol (DAPG) and pyrrolnitrin (PRN) production, probably because the antagonistic bacteria with these antibiotics are more closely related to herbaceous plants in the literature (Hammer *et al.*, 1997; De Souza *et al.*, 2003; Barahona *et al.*, 2010; Bankhead *et al.*, 2016). However, other antifungal compounds different from the *Pseudomonas* spp. compounds produced by the *Enterobacteriaceae*-like group were not taken into account in this study.

Because the production of lytic exoenzymes has been described with implications for biocontrol activity (Haran *et al.*, 1996), we characterized the exoenzymatic production profiles of the bacterial collection. In this characterization, we only detected the production of lipases, proteases and chitinases by the different isolates and not amylases, β-glucanases or cellulases; these exoenzymatic activities are probably related to other groups of soil microorganisms, such as fungi (Haran *et al.*, 1996). A high number of isolates was able to produce lipases and proteases, suggesting the broad distribution of these activities in the soil bacterial community, probably due to their involvement in general metabolism, such as the degradation of lipids (Jaeger *et al.*, 1994) and proteins (Frees *et al.*, 2013). However, a large number of isolates exhibited triple lytic activity, including the production of chitinases, which was in agreement with previous observations of this activity in suppressive soils (Cretoiu *et al.*, 2013) and more specifically in suppressiveness soils induced by the amendment of composted almond shells (Vida *et al.*, 2016).

The PGP-related activities assessed with the plate assays confirmed their presence (ability to solubilize an insoluble phosphate source and synthetize siderophores) in a higher number of *Enterobacteriaceae*-like group isolates that were described as PGPR bacteria in previous studies (Vacheron *et al.*, 2013). The maintenance of an adequate level of mineral nutrients (especially P and the available form of Fe^{3+}) can have a beneficial effect on crop production (Ghosh *et al.*, 2015) and a high concentration of high-affinity siderophores in the rhizosphere can inhibit the growth of fungal pathogens when the Fe^{3+} concentration is low (Lugtenberg and Kamilova, 2009).

Interestingly, most isolates from the *Enterobacteriaceae*-like group were producers of lytic exoenzymes and exhibited PGP-related activities, whereas the putative antibiotic producers were mainly allocated into the *Pseudomonadaceae*-like groups, suggesting the specialization of these groups of bacteria and the presence of different modes of action for biological control.

After the first screening, we selected a group of representative isolates with a wide range of responses to different previous assays. Partial sequencing of the 16S rRNA gene was performed to aid in the preliminary identification of the putative genera to which the strains belonged. The limitation of 16S rDNA partial sequencing for the identification of a bacterial strain at the species level has been demonstrated (Loong *et al.*, 2016). Thus, we could only assign a genus name to nine of the isolates with a correlation between the identity analysis (<95%) and the metabolic pattern.

Additionally, *in vitro* seed growth promotion experiments revealed very low activity (only 2 strains) probably due to the source of the bacterial strains, suggesting that the PGP activity was more related to bacteria directly inhabiting the root environment (Kloepper and Schroth, 1980; Hartmann *et al.*, 2009), and supporting the hypothesis of the presence of different groups of microorganisms in soil related to plant growth promotion and biocontrol traits (Bashan and Holguin, 1998).

The biological control activity of eight selected bacteria on avocado plants showed that the isolation of *Gammaproteobacteria* strains from a composted almond shells suppressiveness-induced soil could represent a strategy for selecting microorganisms with biocontrol ability against *R. necatrix*, potentially *Serratia* spp., *Pseudomonas* spp. and *Stenotrophomonas* spp. The strains belonging to the genera *Serratia* spp. and *Pseudomonas* spp. (isolates '3', '5', '9', '15' and '20') are widely described in the literature due to their diverse plant growth-promoting activities and antagonistic interactions with a broad range of soilborne pathogen (Gkarmiri *et al.*, 2015; Kamou *et al.*, 2016).

5. Conclusion

In this work, we demonstrated that specific representatives from the *Gammaproteobacteria* class isolated from a suppressiveness-induced soil after amendment with composted almond shells displayed biocontrol abilities and associated traits that could be involved in the suppressiveness of the avocado phytopathogenic fungus *R. necatrix.*

CHAPTER IV

Development of a synthetic bacterial consortium to study microbial interactions in the rhizosphere during the biocontrol activity against *Rosellinia necatrix*

Abstract

Induced microbial community from an avocado crop soil after organic amendment, have a key role in suppressiveness against avocado white root rot (WRR) caused by the fungus *Rosellinia necatrix*. This suppressive phenotype is the result of the soil microorganisms activities that do not act as individuals but as a dynamic community where different kind of interactions could take place. However, due to the difficulties of working about the interactions inside natural communities, a promising way to afford such objective is to create artificial microbial communities that could retain the traits of their natural microbiome. In this study, we start the modelling of a bacterial consortium with three biological control agents against WRR, *Pseudomonas chlororaphis* PCL1601, *Pseudomonas chlororaphis* PCL1606 and *Pseudomonas pseudoalcaligenes* AVO110. Stability and compatibility among the members of this artificial consortium have been confirmed. Furthermore, roots visualization assays revealed the colonization pattern of the putative synthetic community and the stability of the bacterial consortium along the avocado and wheat root. Additionally, biological control experiments against *R. necatrix* were performed and confirmed that the bacterial consortium retained the biocontrol activity. In order to further understand microbial interactions that could happen during biocontrol process, the genome of PCL1601 was sequenced, allowing genome comparisons and predictions of secondary metabolites production of the three bacterial partners. In this work, we included the study about production of volatile organic compounds (VOCs) due to the importance in microbial communication and antifungal activity. The results showed that VOCs can constitute a source of antifungal compounds, helping in the biocontrol phenotype, with some of these VOCs produced only when the bacterial assayed were organised as a synthetic community, but not as individual organism.

1. Introduction

Soil microbial suppressiveness can be considered as a direct result of the activities from soil microorganisms, who contribute to all biogeochemical cycles. Most of the soil processes are the result of the microbial activities in their natural environment, where microbes do not act as single individuals, but do it as a dynamically microbial community, where all cells could interact and communicate each with another (Mitri and Foster, 2013). However, it is difficult to stablish what is the role of the different microbes into a natural community. A promising way to overcome the difficulties of studying complex communities is to create artificial microbial consortia that can retain some characters of their natural microbiome to be further studied. These communities could act as a model system to evaluate the role of ecological, structural and functional features of communities in a controlled way (Großkopf and Soyer, 2014).

Recently, special interest have being gained for the microbial community enhanced into an agricultural suppressiveness-induced soil of avocado (*Persea americana* Mill.) against the white root rot (WRR), caused by the soilborne fungus *Rosellinia necatrix*. Studies have demonstrated that the use of composted almond shells as organic amendments or mulches increases soil suppressiveness (Bonilla *et al.*, 2015). In fact, this suppressive activity were directly related with the microbial composition and activities, specifically with the increase of representatives from the bacterial class *Gammaproteobacteria* (including the genera *Pseudomonas*, *Serratia* and *Stenotrophomonas*) and the fungal class *Dothydeomycetes* (Vida *et al.*, 2016).

Each host provides a microhabitat with different abiotic conditions, which directly influence the structure of the root microbial community (Berg *et al.*, 2015). Prevoius studies have described many bacterial strains isolated from soil and rhizosphere of avocado as biocontrol agents against *R. necatrix*, many of them included into *Pseudomonas* spp. genus (Pliego *et al.*, 2012; Vida *et al.*, 2017a under review).

Pseudomonads is a large genus placed into the *Gammaproteobacteria* class, well known for its frequent isolation from soil and rhizosphere environments (Haas and Défago, 2005), utilization of a wide range of organic compounds (Wu *et al.*, 2011) and production of secondary metabolites (Gross and Loper, 2009). Members of the genus *Pseudomonas* (*sensu stricto*) show remarkable metabolic and physiologic versatility, enabling colonization of diverse habitats, and showing potential in biotechnological applications (Silby *et al.*, 2011). Many Pseudomonads from *P. fluorescens*, *P. chlororaphis* and *P. aeruginosa* groups interact with plants in order to contribute in plant health by antagonizing plant-pathogenic microorganisms (biocontrol strains) and directly influencing plant disease resistance and growth (plant growth-promoting bacteria) (Haas and Defago, 2005).

Using the avocado-*R. necatrix* test system, three biocontrol agents have been isolated and well-described in previous works. Two of them, *P. chlororaphis* PCL1601 and PCL1606 were isolated from the rhizosphere of healthy avocado trees and screened for their antagonistic and biocontrol activity against *R. necatrix* (Cazorla *et al.*, 2006). This ability were related with the production of different antimicrobial compounds. *Pseudomonas chlororaphis* PCL1601 produced proteases, lipases, hydrogen cyanide (HCN), phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) whereas *P. chlororaphis* PCL1606 produced proteases, lipases, siderophores, HCN, pyrrolnitin (PRN) and 2-hexyl 5-propyl resorcinol (HPR) antifungal compounds, crucial for biological control against *R. necatrix* and for avocado root colonization (Cazorla *et al.*, 2006; Calderón *et al.*, 2014). A third bacterial strain is *P. pseudoalcaligenes* AVO110, also isolated from roots of healthy avocado trees, but selected because its ability to efficiently colonize avocado roots, and also displaying biocontrol towards *R. necatrix*. Previous studies showed that the mode of action of this strain could be related with the competition for niches and nutrients because it cannot

produce any well-known antifungal metabolites from Pseudomonads (Pliego *et al.*, 2007; 2008).

The construction of an artificial bacterial consortium with these characterized strains could represent an approach to understand how cells live in close proximity, and to unravel their interaction during the biocontrol activity. In this sense, colonization of root system have been described in literature as an essential trait for further applications (de Weert *et al.*, 2002), including biocontrol of soilborne diseases (Chin-A-Woeng *et al.*, 1998; Lugtenberg and Kamilova, 2009; Barahona *et al.*, 2010). In this environment, the efficient root colonisers can compete for niches and nutrients in the rhizosphere and promote the production of antifungal compounds, so the knolewdge of the colonization patterns of an artificial consortium is crucial in order to evaluate them from an ecological and biotechnological point of view (De Roy *et al.*, 2014).

Because these different biocontrol *Pseudomonas* spp. displayed different phenotypes, it is not surprising that its diversity could extends to the genomic sequence level. Bacterial genome sequences can now be generated faster and cheaper enough to be considered part of the toolbox for investigating bacteria (Edwards and Holt, 2013). Furthermore, this technology can provide, for example, insight into mode of life and essential processes of bacterial strains. The analysis of core sequences among bacteria or regions that are unique to a specific strain could help us to unravel the social interactions established between microorganisms. The genome comparative analysis could provide information about the hypothetical role of the different strains during biocontrol process, analysing the putative secondary metabolites production and activities implicated in biogeochemical cycling of nutrients and inter- and intraspecific interactions (Silby *et al.*, 2011).

Additionally, important molecules in communication between bacteria are the volatile organic compounds (VOCs), commonly produced by bacteria and emitted to environment. Many VOCs play a significant role in the communication between

organisms, affecting to growth, antibiotic production and gene expression of soil bacteria (Garbeva *et al.*, 2014). Volatile molecules can diffuse through liquid and gaseous phases of the soil (Effmert *et al.*, 2012), playing key roles in interspecific bacterial interaction physically separated in the porous soil matrix. Concretely, *Pseudomonas* spp. have been described to produce different VOCs that inhibit the growth of different soilborne pathogens (Kai *et al.*, 2009). Historically, most studied is the compound hydrogen cyanide (Knowles, 1976), volatile compound that inhibits several metal-containing enzymes such as cytochrome c oxidase of the respiratory chain (Effmert *et al.*, 2012). For this reason, HCN could act as a toxic for most aerobic organisms living in the same habitat as Pseudomonads. *Pseudomonas chlororaphis* PCL1601 and PCL1606 have been described to produce HCN (Cazorla *et al.*, 2006), but *P. pseudoalcaligenes* AVO110 is a non-producer of this volatile (Pliego *et al.*, 2007). However, nothing is known about the production of other VOCs from these strains.

In this study, in order to inititate a broader study on the interaction that take place on the rhizosphere during the biocontrol against *R. necatrix*, we initiated the construction and study of a 3-bacterial consortium biocontrol strains previously isolated from an avocado agricultural soil. First, absence of negative interactions among them were confirmed by compatibility plate assays, and visualization of spatial distribution in root surface of the microbial consortium in presence or absence of the pathogen *R. necatrix*. The results confirmed the stability of the artificial consortium on avocado roots. Additionally, the biocontrol activity of the microbial consortium was evaluated in order to confirm the retainment of such trait. Furthermore, to start the study on the synthetic community, genome comparison of the three *Pseudomonas* spp. strains (*P. chlororaphis* PCL1601 and PCL1606 and *P. pseudoalcaligenes* AVO110) were performed, as well as *in silico* genome search of putative secondary metabolites production. As first step to study their interaction and, due to the importance of the

production of secondary metabolites both in antagonistic activity and microbial interactions, the analysis of volatiles organic compounds (VOCs) emitted by the bacterial consortium have been initiated.

2. Materials and methods

2.1 Microorganisms, culture and conditions

The plasmids, bacterial and fungal strains used in this study are listed in Table 9. Luria Bertani (LB) medium was used to growth the strains at 25ºC (Bertani, 1951). When using transformed strains, media were supplemented with tetracycline (50 µg/ml), trimethoprim (2 mg/ml) or gentamicin (20 µg/ml). The bacteria were stored at -80ºC in LB with 15% glycerol.

Rosellinia necatrix CH53-GFP (Pliego *et al.*, 2012) was used in this study and was grown in potato dextrose agar (PDA) supplemented with hygromicin B (10 µg/ml) at 25ºC. Fungus was stored at 4ºC as previously described (Gutierrez-Barranquero *et al.*, 2012).

2.2 *In vitro* dual-culture experiments

Compatibility plate assays

The three wild type *Pseudomonas* spp. strains (PCL1601, PCL1606, AVO110) were evaluated for their compatibility *in vitro* using dual cultures. We also used the biocontrol strain *Bacillus subtilis* PCL1608 (Cazorla *et al.*, 2007) as a reference for such analysis. Five-millilitre cultures on LB medium of PCL1601, PCL1606, PCL1608 and AVO110 were overnight incubated at 25ºC and 150 rpm. Petri dishes with LB and TPG (Calderón *et al.*, 2014) diluted 1/20 agar were surface inoculated with one of the strains and dropped with 10 µl of a culture of 10^9 cfu/ml of each other

tested bacteria over a sterile disc. Plates were sealed and upright incubated during 2-3 days at 25ºC in dark and growth inhibition halo checked for negative interactions.

AHL reporter plate assays

The production of N-acyl homoserine lactones as autoinducers of *quorum sensing* (QS) by individual *Pseudomonas* spp. strains and the artificial bacterial consortium was evaluated (McClean *et al.*, 1997, with modifiations). Five-millilitre cultures on LB medium of *P. chlororaphis* PCL1601, PCL1606, *P. pseudoalcaligenes* AVO110 and *Chromobacterium violaceum* CV026 (McClean *et al.*, 1997) were overnight incubated at 25ºC and 150 rpm. After that, LB plates were surface inoculated with *C. violaceum* CV026 and air-dried. Then, sterile paper discs were placed over the *C. violaceum* CV026 inoculated plates and soaked with 10 µl of the *Pseudomonas* spp. cultures (10^9 cfu/ml) and microbial consortium cultures (PCL1601, PCL1606, AVO110 bacterial isolates mixed in a ratio 1:1:1). Plates were sealed and upright incubated during 2-3 days at 25ºC in dark. Finally, plates were examined for the stimulation of violacein synthesis (indicated by blue/purple pigmentation of the bacterial around the paper disc) and colored halos measured.

Table 9: Bacterial and fungal strains used in this study.

	Lytic exoenzymes				Antibiotics				Antagonism						Reference
Bacterial strains:	**Cel**	**Glu**	**Pro**	**Lip**	**HCN**	**PCA**	**HPR**	**PRN**	***R.n.***	***P.c.***	***R.s.***	***P.u.***	***S.r.***	***F.o.***	
Bacillus subtilis PCL1608	□	■	■	□	□	□	□	□	■	■	■	■	■	■	Cazorla et al., 2007
Chromobacterium violacearum CV026	■	■	■	■	■	■	■	■	■	■	■	■	■	■	McClean et al., 1997
Pseudomonas chlororaphis PCL1601	□	□	■	■	■	■	□	□	■	■	■	■	■	■	Cazorla et al., 2006
Pseudomonas chlororaphis PCL1606	□	□	■	■	■	□	■	■	■	■	■	■	■	■	Cazorla et al., 2006
Pseudomonas pseudoalcaligenes AVO110	■	□	□	□	□	□	□	□	■	□	■	■	■	□	Pliego et al., 2007
Fungal strains:															
Rosellinia necatrix CH53-GFP	CH53 derivative by insertion of the plasmid pCPXHY1eGFP, HygBR														Pliego et al., 2012
Plasmids:															
pJA-dsred	pBRJ-H plasmid derivative containing dsred gene production, Tet^r														Abd-Alla et al., 2004
pIN69-Zsyellow	pIN69 plasmid derivative containing Zsyellow gene production, Tmp^r														Vergunst et al., 2010
pBAH-8-GFP	pBBR1MCS-5 plasmid derivative containing GFP gene production, Gm^r														Huber et al., 2002

Black box: positive result; white box: negative result; grey box: no determined; cel: cellulase; glu: β-glucanase; pr:, protease; lip: lipase; HCN: hydrogen cyanide; PCA: phenazine-1-carboxylic acid; HPR: 2-hexyl 5-propyl resorcinol; PRN: pyrrolnitrin; R.n.: *Rosellinia necatrix*; P.c.: *Phytophthora cinnamomi*; R.s.: *Rhizoctonia solani*; P.u.: *Pythium ultimum*; S.r.: *Sclerotium rolfsii*, F.o.: *Fusarium oxysporum* f.sp. *radicis-lycopersici*; Tet: tetracycline; Tmp: trimethoprim; Gm: gentamicine, HygB:hygromicine B

2.3 Stability on avocado and wheat roots

Stability of the bacterial consortium on roots was evaluated by confocal laser scanning microscopy (CLSM), and data complemented with bacterial counts. Two different plants (avocado and wheat) were used, in order to facilitate further comparisons.

Construction of tagged strains

To facilitate visualization of bacterial strains on roots, fluorescent-labelled derivatives were constructed for each bacterial component of the consortium. To obtain bacterial strains expressing fluorescent proteins, the corresponding plasmids (Table 9) were incorporated into each wild type *Pseudomonas* spp. strains using standard electroporation methodology (Choi *et al.*, 2006). For construction of competent cells, bacterial cultures (200 ml) in 2xTY medium (16 g Tryptone-Peptone; 10 g yeast extract; 5 g NaCl per liter) at OD_{600nm}=0.5 from each strain were centrifugated (4000 rpm, 8 min, 4ºC) and the pellets washed with glycerol solution 10% and stored at -80ºC.

For electroporation, 500 ng of plasmid purified using GeneJET plasmid miniprep kit from Thermo Fisher Scientific (San Jose, California, USA) was mixed with 50 µl of competent cells (10^9 cfu/ml). The mixture was transferred to a 2 mm gap width electroporation cuvette and a pulse applied (Ω 2.5 kV on a MicroPulser™; Bio-Rad). One milliliter of room temperature SOC medium (20 g Trypton-Pepton; 5 g yeast extract; 4.8 g MgSO4; 3.6 g glucose; 0.5 g NaCl; 0.2 g KCl per liter) was added and cells transferred to a 1.5 ml tube for 90 min at 25 °C. Then, cells were plated on an LB supplemented plate with the corresponding antibiotic (tetracycline 50 µg/ml; trimethoprim, 2 mg/ml; gentamicin, 20 µg/ml). The plates were incubated at 25 °C until colonies appeared recovered in pure culture and the fluorescent character checked using a Molecular Imager ChemiDoc system (Bio-Rad).

Root colonization assays

To perform the root visualization assays, commercial avocado plants and wheat seedlings were disinfected, washed and bacterized using fluorescent-labelled bacteria suspension (10^8 cfu/ml). Avocado plants and wheat seeds incubated with sterile LB medium were used as control. These experiments were carried out at the same time for each plant-pathogen system potting soil with and without infecting with *R. necatrix* CH53-GFP. Assays were performed in a growth chamber at 25°C and 70 % relative humidity, with 16 h of daylight.

After 10 and 20 days of growth, the wheat plants were carefully removed from the soil, and pieces of roots were placed directly on glass slides and examined using a Leica confocal system equipped with detectors and filter sets that simultaneously monitored red (excitation 563 nm/ emission 582 nm), green (excitation 488 nm/ emission 507 nm) and yellow (excitation 529 nm/ emission 539 nm) fluorescence. We selected these times in order to equilibrate the time between strains establishment and fungal root degradation (approximately at 35 days).

In the same way, after 7 and 14 days of growth, the avocado plants were carefully removed from the soil, and pieces of roots were placed directly on glass slides and examined using CSLM microscopy. At these times, we could observe the bacterial root colonization before the start of fungal root degradation (approximately at 21 days).

Furthermore, one gram of roots tissues was place into a stomacher bag containing 2 ml of sterile saline solution (0.8% NaCl). Samples were homogenezed during 4 min in a stomacher® and 10-fold serial dilution were performed. Then, 100 µl of each dilutions were inoculated in LB plates supplemented with the corresponding antibiotic, and incubated at 25ºC overnight.

2.4 Biocontrol assays against avocado white root rot

In order to confirm that the biocontrol trait still present in the artificial consortium, biological control experiments were performed. Biocontrol assays against white root rot were performed both using the avocado-*R. necatrix* system (Cazorla *et al.*, 2006) and wheat-*R. necatrix* systems (Vida *et al.*, 2016) and derivatives strains individually or as microbial consortium. The biocontrol assays were performed with six-month-old commercial avocado plants obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain) and 3 days germinated wheat seeds. The roots from the avocado plants and/or germinated wheat seedlings, were disinfected by immersion in 0.1% NaOCl for 20 min and then washed (20 min) with sterile distilled water. Roots and wheat seedling were then bacterized by inmersion in a suspension (10^8 cfu/ml) of individual bacterial isolates or with the microbial consortium (PCL1601, PCL1606, AVO110 bacterial isolates mixed in a ratio 1:1:1) for 20 min. Avocado plants were placed into square plastic pots and wheat seedlings were placed into plastic seedling trays both containing potting soil. Fungal infection with *R. necatrix* was performed using wheat grains as previously described (Freeman *et al.*, 1986; Vida *et al.*, 2016). Avocado plants and wheat seeds incubated with sterile LB medium were used as control. Three sets of fifteen avocado plants and fifty wheat seedlings were tested per treatments. The plants were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight, with watering twice per week. Aerial symptoms of the WRR disease were recorded on a scale of 0–3, and a disease index (DI) was calculated at the end of the assay (Cazorla *et al.*, 2006).

The biocontrol assays data were statistically analyzed using an analysis of variance (Sokal and Rohlf 1986), followed by Fisher's least significant difference test ($p<0.05$) using the SPSS 22 software (SPSS Inc., Chicago).

2.5 DNA extraction and sequencing

To proceed with the genome sequence analysis of the bacterial components of the consortium, a draft genome of PCL1601 was obtained (available, NCBI's database accession number MSCT00000000.1, Vida *et al.*, 2017b) to be finally compared with the genome of PCL1606 (available, NCBI's database accession number CP011110, Calderón *et al.*, 2015) and AVO110 (draft genome obtained but not published yet).

Genomic DNA from *Pseudomonas chlororaphis* PCL1601 was extracted from bacterial cells growing in an overnight culture in LB medium at 25ºC and 150 rpm using the UltraClean® Microbial DNA Isolation kit (Mo Bio laboratories, Inc. CA, USA). To verify DNA quality, polymerase chain reaction (PCR) amplifications were performed in a final volume of 25µl, which contained 10 µM each primer (341F-907R to amplify partial sequence of 16S rRNA gene), 2.5 U of GoTaq Flexi DNA polymerase (Promega Corp., Madison, WI, USA), dNTP mixture (250 µM each dNTP), 2 mM MgCl2, 1x GoTag Flexi buffer, and approximately 50 ng of template DNA. The PCR amplification conditions consisted of an initial denaturation for 2 min at 94ºC; followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 61ºC for 1 min, and extension at 72ºC for 1 min; and then a final extension at 72ºC for 10 min. The PCR products were analysed for size and quantity by electrophoresis on 1% agarose gels in Tris-acetate-EDTA buffer. Ethidium bromide was used at a concentration of 1 µl/ml to visualize the DNA bands.

The genome of *P. chlororaphis* PCL1601 was sequenced by ChunLab, Inc (Seoul, Korea) using PacBio (20K) sequencing platform and assembled using PacBio SMRT Analysis 2.3.0. Contings were ordered using the reference genome of *P. chlororaphis* PA23 (NCBI's database accession number CP008696.1). The genome of *P. chlororaphis* PCL1601 was automatically annotated using the services of ChunLab. Manual curation and the comparative analysis was performed using the CLgenomics v. 1.53 software.

2.6 Genome sequence comparison

Genomes of *P. chlororaphis* PCL1601, *P. chlororaphis* PCL1606 and *P. pseudoalcaligenes* AVO110 were compared. Core and unique genes among compared strains were obtained using the EzBioCloud comparative genomics tools (http://cg.ezbiocloud.net/) and CLgenomics® software from ChunLab, Inc. Secondary metabolite production clusters were examined using the antiSMASH program (http://antismash.secondarymetabolites.org/).

2.7 Identification of volatiles compounds produced

In order to gain insight into the putative role of volatiles organic compounds (VOCs) produced by the three *Pseudomonas* spp. strains alone and when they are together, its production was analized.

Antifungal activity

Compartmentalized plates, one half filled with LB agar and the other half with PDA, were used to perform dual plate antagonistic activity by VOCs production. The LB agar was inoculated with a bacterial individually or the consortium using 10 µl drops of 10^9 cfu/ml LB medium culture (PCL1601, PCL1606, AVO110 bacterial isolates mixed in a ratio 1:1:1). Agar disks (6 mm diameter) of 5-days old fungal mycelium of *R. necatrix* CH53-GFP were used to inoculate PDA half-plate. Plates were tightly sealed with parafilm and incubated at 25ºC during 3 days. Plates without bacterial inoculation were used as control. After incubation time, fungal growth area where calculated using a Molecular Imager ChemiDoc system (Bio-Rad).

GC/MS analysis

To identify the VOC present in each culture, bacterial strains were grown in 10 ml LB agar using 20 ml glass head-space bottles (Chromtech, Idstein, Germany). The isolates were inoculated using a loop of an individual or parallel streaks of the three bacterial

strains. After 18 hours of growing at 25ºC, vials were sealed and incubated for additional 2 h. Solid phase micro extraction (SPME) and gas chromatography-mass spectrophotometry (GC-MS) were performed as previously described by Cernava *et al.*, (2015). Obtained spectra were compared with NIST Mass Spectral Database and specific compounds identified based on their Kovats retention indices and comparison to reference substances (ChemSpider database from Royal Society of Chemistry; http://www.chemspider.com).

3. Results

3.1 Dual-plate assays

Compatibility plate assays

The strains of *Pseudomonas* spp. used in this study were compatible each other in dual plate assays performed in LB and TPG 1/20 media without any differences in response depending of the used culture media. Moreover, these strains were able to surface growth when we inoculated a sterile paper disc with *B. subtilis* PCL1608 culture. Nevertheless, PCL1608 inhibited its surfaced growth around bacterial disc of *Pseudomonas* spp. strains PCL1601, PCL1606 and AVO110 (Table 10, Figure 22A).

Table 10: Compatibility of tested strains by plate assay. The growth of different strains were performed in LB and TPG diluted media. Strains on the top row were dispersed by the surface and strains on the left column were dropped in a sterile paper disc (10 µl) over dispersed.

	Surface inoculated bacteria			
Disc inoculated bacteria	**PCL1601**	**PCL1606**	**AVO110**	**PCL1608**
PCL1601	■	●	●	○
PCL1606	●	■	●	○
AVO110	●	●	■	○
PCL1608	●	●	●	■

Black circle: inoculated surface growth; White circle: growth inhibit on inoculated surface; PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P.chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110; PCL1608: *Bacillus subtilis* PCL1608.

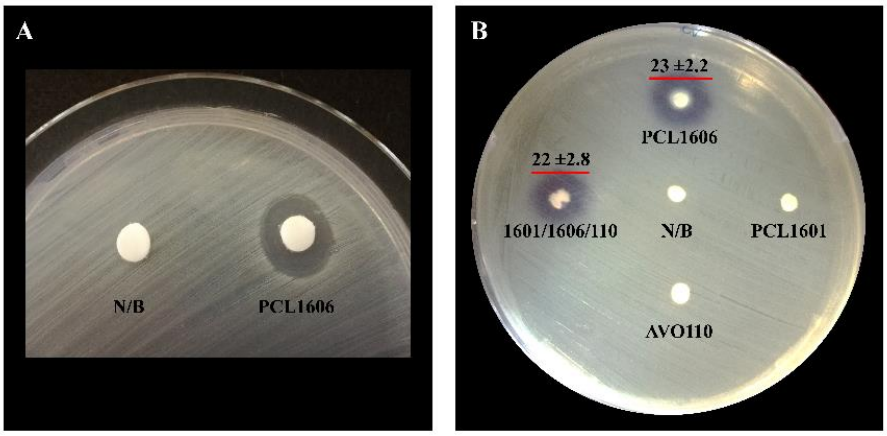


Figure 22: Dual-plate assays. **(A)** Compatibility plate assay: growth inhibition halo (right) of *Bacillus subtilis* PCL1608 dispersed by the surface caused by *Pseudomonas chlororaphis* PCL1606 dropped in a sterile paper disc (10 µl). **(B)** AHLs (acyl homoserine lactones) reporter plate assay: violacein produced by *Chromobacterium violaceum* CV026 induced by the bacterial production of AHLs (presence of violet halo) or not. CV026 was spreaded by the surface and cultures of *Pseudomonas* spp. strains and bacterial consortium were dropped in a sterile paper disc (10µl). Red line, diameter of halo in millimeter ± standar desviation.
PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P. chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110; 1601/1606/110: microbial consortium; N/B, negative control inoculated with sterile LB broth.

AHL production plate assays

In this study, we evaluated the presence of *quorum sensing* molecules by *Pseudomonas* spp. strains and microbial consortium using the biosensor for AHLs *Chromobacterium violaceum* CV026. The results showed that only PCL1606 was able to induce the violacein production by CV026 (Figure 22B). This violacein production was also induced by the microbial consortium, without significantly difference in the halo diameter between both treatments.

3.2 Colonization patterns of *Pseudomonas* spp. on root

The colonization patterns of the labeled bacterial strains *P. chlororaphis* PCL1601 (DsRed), *P. chlororaphis* PCL1606 (ZSYellow) and *P. pseudoalcaligenes* AVO110 (GFP) on wheat and avocado roots were monitored individually and forming an artificial microbial consortium under confocal laser scanning microscopy (CLSM). After inoculation of seedlings wheat with individual strains or consortium, in presence of pathogen or not, we observed their establishment on the rhizosphere. These assays allowed us to distinguish two different colonization patterns: single cells or mixed networks covering the root surface. When *Pseudomonas* spp. strains were inoculated individually, mainly, single cells were observed along roots. However, when the three strains consortium was inoculated we observed mixed networks of bacteria (Figure 23A). Bacterial counts at 10 and 20 days post-inoculation, showed a reduction of 1 order of magnitude in the number of cfu/ml for PCL1601, whereas PCL1606 and AVO110 kept their number of cfu/ml practically stable (Table 11A, C). In microbial consortium, only AVO110 showed the same number of cells at two times, whereas PCL1601 and PCL1606 reduced their presence in 1 order of magnitude.

Similarly, after inoculation of avocado roots with individual strains or the bacterial consortium (with or without *R. necatrix*), the two rhizosphere colonization patterns were also noted. In this case, the number of cfu/ml maintained stable along

experiments, with similar results at 7 and 14 days post-inoculation (Table 11B, D). However, denser and organized networks were observed at 14 days after inoculation with the consortium, forming a biofilm-like structure around root cells (Figure 26B). Surprisingly, the red-labelled PCL1601 cells appeared to be all of them below the green- and yellow- tagged cells.

Table 11: Plate counts of *Pseudomonas* spp. strains during roots colonization assays. **(A)** Bacterial counts in wheat roots colonization assays at 10 and 20 days growth in soil inoculated with *Rosellinia necatrix*. **(B)** Bacterial counts in avocado roots colonization assays at 7 and 14 days growth in soil inoculated with *Rosellinia necatrix*. **(C)** Bacterial counts in wheat roots colonization assays at 10 and 20 days growth in soil not inoculated with *Rosellinia necatrix*. **(D)** Bacterial counts in avocado roots colonization assays at 7 and 14 days growth in soil not inoculated with *Rosellinia necatrix*.

Soil inoculated with *R. necatrix*

A

Wheat roots	10 days	20 days
PCL1601	7.41 ± 0.45	6.29 ± 0.89
PCL1606	5.56 ± 0.65	5.28 ± 0.56
AVO110	5.31 ± 0.12	5.61 ± 0.46
Microb. Conso.		
PCL1601	5.08 ± 0.65	4.31 ± 0.52
PCL1606	5.41 ± 0.56	4.72 ± 0.87
AVO110	5.26 ± 0.48	5.28 ± 0.92

B

Avocado roots	7 days	14 days
PCL1601	6.22 ± 0.25	6.49 ± 0.35
PCL1606	6.40 ± 0.33	5.83 ± 0.18
AVO110	6.75 ± 0.74	6.18 ± 0.20
Microb. Conso.		
PCL1601	5.15 ± 0.85	5.33 ± 0.69
PCL1606	6.53 ± 0.13	5.96 ± 0.45
AVO110	5.85 ± 0.21	5.83 ± 0.85

Soil not inoculated with *R. necatrix*

C

Wheat roots	10 days	20 days
PCL1601	6.31 ± 0.16	5.98 ± 0.95
PCL1606	5.89 ± 0.33	5.56 ± 0.46
AVO110	5.48 ± 0.42	5.15 ± 0.72
Microb. Conso.		
PCL1601	4.98 ± 0.19	4.79 ± 0.18
PCL1606	5.13 ± 0.48	4.87 ± 0.74
AVO110	5.07 ± 0.78	5.10 ± 0.12

D

Avocado roots	7 days	14 days
PCL1601	6.01 ± 0.15	6.19 ± 0.27
PCL1606	6.40 ± 0.42	6.02 ± 0.42
AVO110	6.06 ± 0.11	6.30 ± 0.87
Microb. Conso.		
PCL1601	5.91 ± 0.65	5.18 ± 0.26
PCL1606	4.98 ± 0.24	4.76 ± 0.54
AVO110	5.13 ± 0.37	4.96 ± 0.76

PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P.chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110; Microb. Conso.: microbial consortium formed by PCL1601, PCL1606 and AVO110. Data are presented as $\log_{10}$ cfu/ml ± standard deviation

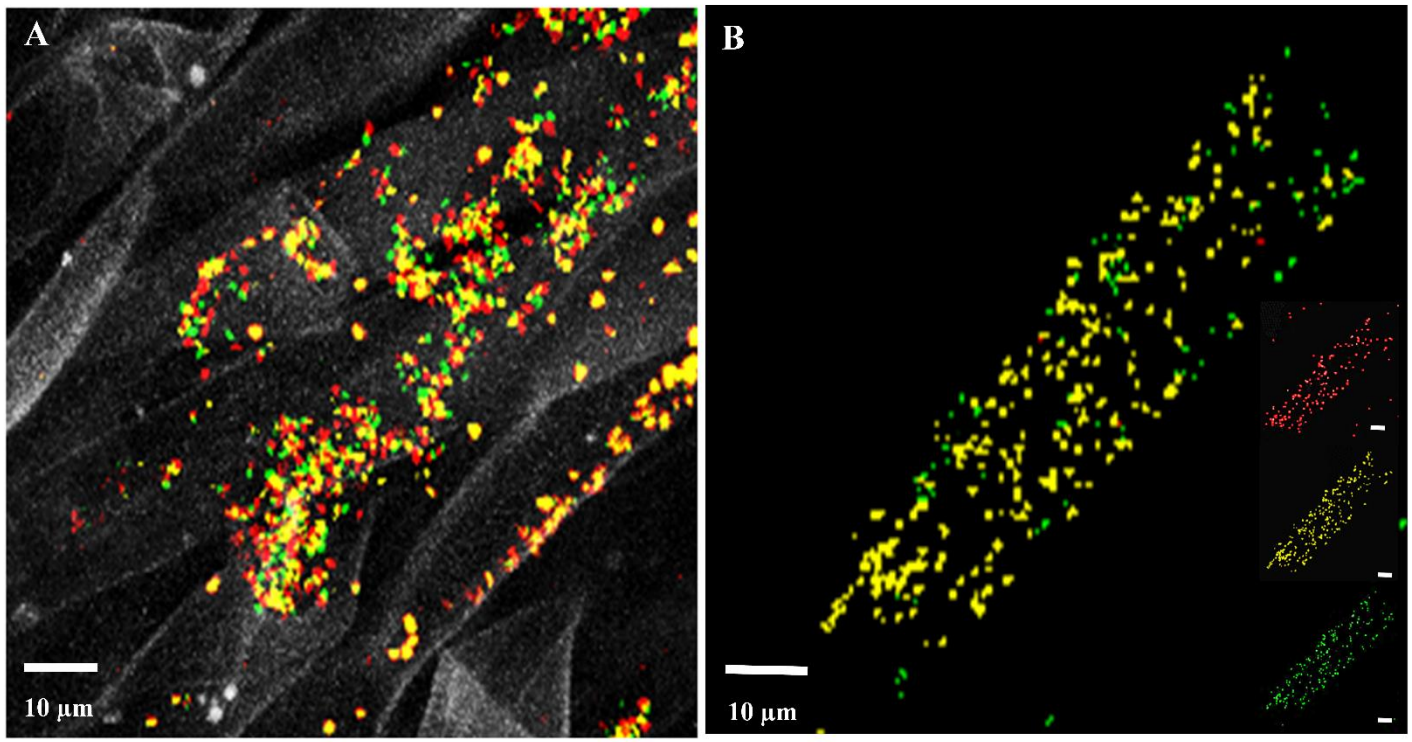


Figure 23: *In situ* visualization of tagged bacterial roots colonization assays. Wheat seedlings and avocado plants were inoculated with the bacterial strains (*Pseudomonas chlororaphis* PCL1601-RFP, *P.chlororaphis* PCL1606-YFP, *P. pseudoalcaligenes* AVO110-GFP) as microbial consortium (*Pseudomonas* spp. strains mix at same concentration) before being transferred to potting soil. Scanning confocal laser microscopy allowed visualization of red, yellow and green fluorescence emitted by the bacteria. **(A)** Observed colonization pattern of microbial consortium at 10 days in wheat roots. **(B)** Observed colonization pattern of microbial consortium at 14 days in avocado roots. Right down, same picture with the three different wavelength used in this study.

3.3 Biocontrol assays

Biocontrol assays using avocado-*R. necatrix* and wheat-*R. necatrix* systems were conducted as previously described. In wheat-*R. necatrix* system, when no bacteria were applied to wheat seeds, the plants showed a disease index of 76% (Figure 24). In this case, bacterial isolates PCL1601, PCL1606 and AVO110 and microbial consortium (PCL1601-PCL1606-AVO110) shown a disease index of 74%, 68%, 75% and 64% respectively, but not significantly lower than control treatment without bacterization.

However, in avocado experiments, when no bacteria were applied to the roots, the plants infected with *R. necatrix* showed a disease index of 73% (Figure 24) after 21 days post-inoculation. However, when *P. chlororaphis* PCL1601, *P. chlororaphis* PCL1606 and *P. pseudoalcaligenes* AVO110 strains were applied to the roots, disease index was reduced significantly (PCL1601 for 39%, PCL1606 for 44%, AVO110 for 46%). Then, after application of microbial consortium, the disease index was significantly reduced (39%) regarding control treatment, but no significant differences were observed with single cells treatments.

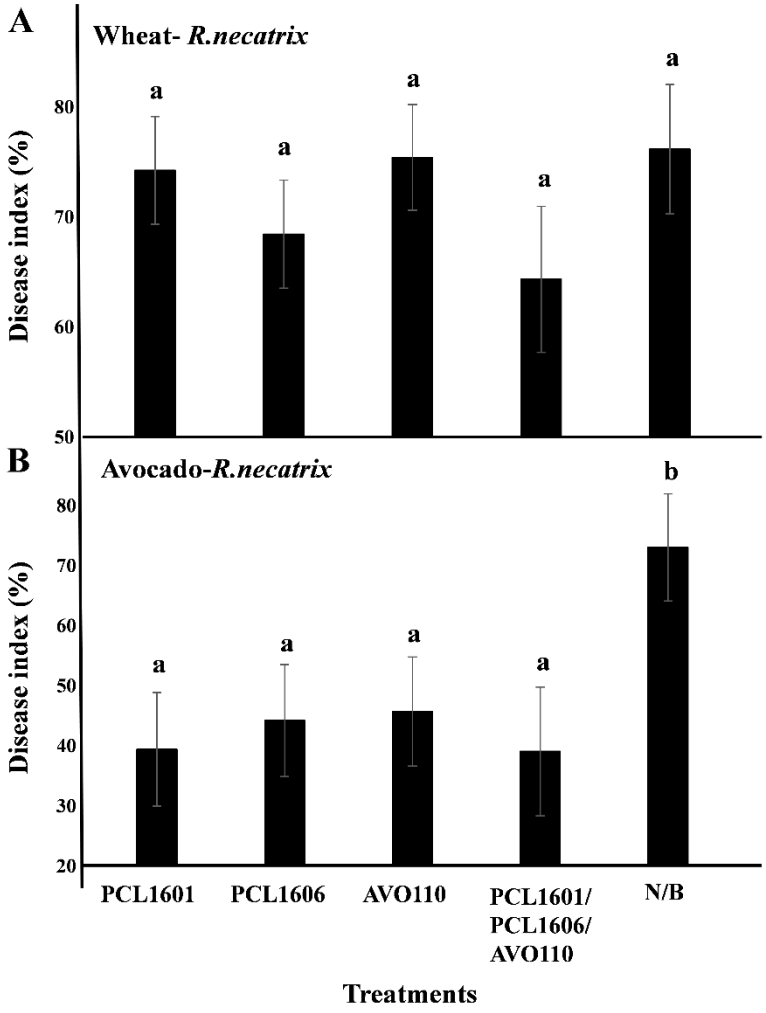


Figure 24: Biocontrol of *Rosellinia necatrix*-induced white root rot in wheat and avocado plants. **(A)** Wheat plants were scored as sick or healthy at 35 days after bacterization. **(B)** Avocado plants were scored as sick or healthy at 21 days after bacterization. Data were analyzed for significance using an arcsine square root transformation with analysis of variance followed by Fisher's least significant difference test ($p<0.05$). Values with different letter indications denote a statistically significant difference. PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P.chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110; 1601/1606/110: microbial consortium; N/B: negative control.

3.4 Genome features

In this study, we obtained a draft sequence of the *P. chlororaphis* PCL1601 genome, by using PacBio 20K procedure. The obtained draft genome showed the following features: sequence was 6,755,445 bp in length, arranged in 25 contigs, which was assembled as a pseudochromosome using *P. chlororaphis* PA23 as template genome (Table 12).

The genome features for each of the 3 *Pseudomonas* spp. strains used in this study are summarized in Table 12. *Pseudomonas chlororaphis* PCL1601 and *P. chlororaphis* PCL1606 have a quite similar size (6.76 and 6.67 Mbp, respectively) and *P. pseudoalcaligenes* AVO110 presented a genome size smaller, with 4.97 Mbp. *Pseudomonas pseudoalcalignes* AVO110 has the highest value for G+C content (64.94%), whereas *P. chlororaphis* strains has a similar value (around 64%). The number of coding sequences (CDS) was higher in PCL1606 genome (6107), closely followed by PCL1601 (5923), and AVO110 (4475) indicating substantial variations between *P. chlororaphis* strains, but higher with *P. pseudoalcaligenes* AVO110 strain.

Table 12: General genomes features of the studied *Pseudomonas* spp.

	PCL1601	**PCL1606**	**AVO110**
	Pseudomonas chlororaphis PCL1601	*Pseudomonas chlororaphis* PCL1606	*Pseudomonas pseudoalcaligenes* AVO110
Status	Assembly(25)	Assembly(2)	Assembly(3)
Genome size	6,755,445	6,662,896	4,970,700
GC content	64.05	64.01	64.94
CDS	5,923	6,107	4,475
rRNA	18	16	15
tRNA	69	71	67
Contigs	25	2	3
Method	PacBio_20K	Illumina_HiSeq_100_PE	Illumina_HiSeq_100_PE
Assembler	PacBio SMRT Analysis 2.3.0	SOAP denovo	SOAP denovo

PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P.chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110.

3.5 Comparative analysis of *Pseudomonas* spp. genomes

CLGenomic software (ChunLab, Inc) was used to obtain the distribution of eggNOG (public resource of orthologous groups of genes) categories for each strains (Huerta-Cepas *et al.*, 2016). The results (Figure 25) showed highly similar distributions (differences below 0.6%) of eggNOG categories between genome strains related with metabolism and cellular regulation (energy production, cell cycle control, nucleotide metabolism, cell wall membrane, inorganic ion transport and production of secondary metabolites). Nevertheless, we observed slight differences in percentage of orthologous genes higher than 1% between genome strains. Percentage in categories such as carbohydrate metabolism and transcription related genes were higher in PCL1601 and PCL1606 genomes, as well as, replication and recombination, translation and ribosomal biogenesis, signal transduction mechanisms and postranslational modification percentage categories were higher in AVO110 genome. At last, we observed a higher number of function unknown genes in PCL1601 genome. Using EzBioCloud Comparative Genomics tools, we detected 8057 POGs (Pan-genome Orthologous Groups) clustered orthologous groups of genes that were identified from the three diferential genomes in different categories (Figure 26). In this case, *P. chlororaphis* PCL1601 presented 5899 POGs detected from 5923 CDS contained in their sequence (n=24 singletons, single CDS sequence that not grouped in any POG category)*; P. chlororaphis* PCL1606 presented 5893 POGs from 6107 CDS (n=214 singletons) and *P. pseudoalcaligenes* AVO110 obtained 4437 POGs from 4475 CDS (n=38 singletons). Venn diagram analysis (Bardou *et al.*, 2014) showed that 36% of the clustered orthologous genes in pan-genomes (POGs) were presented in the three analyzed strains (n=2888), forming the core genome of these species. Moreover, we observed that 29% of POGs were at least in 2 of the 3 strains, mainly shared by PCL1601 and PCL1606 strains (n=2232, 27%; Figure 26A).

A

Name	Putative function	PCL 1601	PCL 1606	AVO 110
C	Energy production and conversion	5.52	6.07	6.00
D	Cell cycle control. cell division. chromosome partitioning	0.61	0.83	0.97
E	Amino acid transport and metabolism	9.36	10.70	9.96
F	Nucleotide transport and metabolism	1.71	2.19	1.79
G	Carbohydrate transport and metabolism	4.80	5.33	2.87
H	Coenzyme transport and metabolism	2.58	3.74	3.74
I	Lipid transport and metabolism	3.57	5.03	5.16
J	Translation. ribosomal structure and biogenesis	3.29	3.97	4.53
K	Transcription	8.80	9.64	6.93
L	Replication. recombiantion and repair	2.42	2.78	3.77
M	Cell wall/ membrane/ envelope biogenesis	5.36	5.71	5.95
N	Cell motility	1.27	3.55	3.90
O	Postranslational modification. protein turnover. chaperones	3.18	3.72	4.32
P	Inorganic ion transport and metabolism	6.35	6.31	5.77
Q	Secondary metabolites biosynthesis. transport and catabolism	2.16	2.02	2.48
S	Function unknow	29.51	23.17	19.57
T	Signal transduction mechanisms	5.85	5.25	7.82
U	Intracellular trafficking. secretion and vesicular transport	2.44	0.00	3.29
V	Defense mechanisms	1.24	0.00	1.19

B

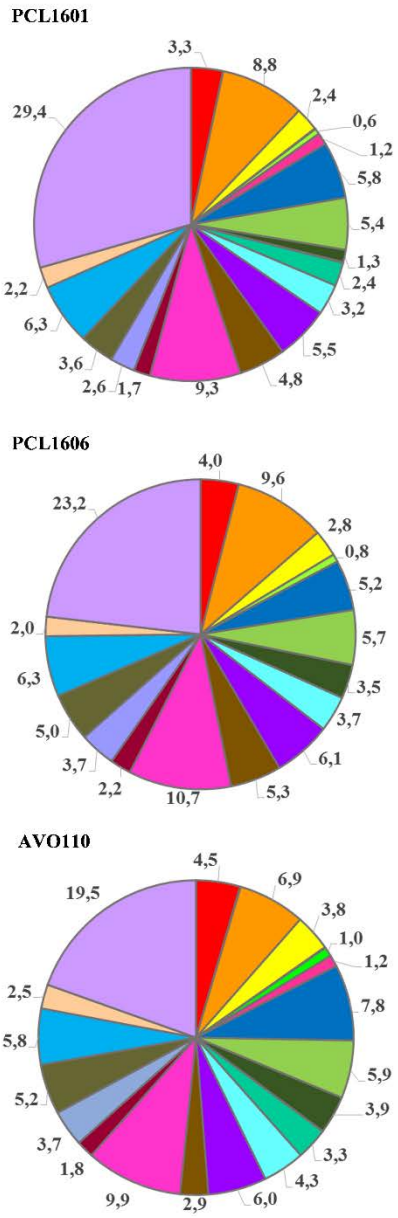
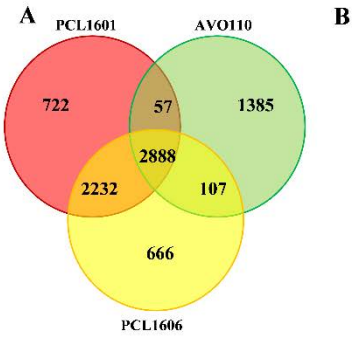
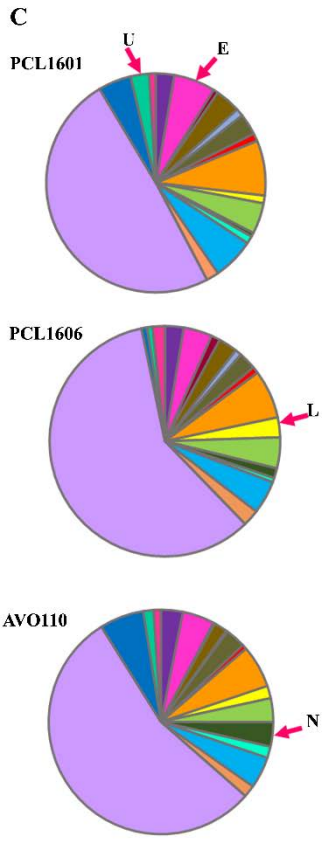


Figure 25: Comparative analysis of the functional ategories based on cluster of orthologous groups of the protein-coding genes of the *Pseudomonas* spp. strains**. (A)** List of analized categories with a percentage higher than 1% in at least of the strains. (**B)** Distribution of studied categories for each strain. These results were generated using CLGenomics software. PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P. chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110.





B

Putative funcion		PCL 1601		PCL 1606		AVO 110	
		n	%	n	%	n	%
Energy production and conversion	C	19	2.7	17	2.6	42	3.1
Amino acid transport and metabolism	E	43	6.1	26	4.0	62	4.6
Nucleotide transport and metabolism	F	5	0.7	8	1.2	3	0.2
Carbohydrate transport and metabolism	G	26	3.7	17	2.6	25	1.9
Coenzyme transport and metabolism	H	8	1.1	6	0.9	3	0.2
Lipid transport and metabolism	I	22	3.1	16	2.5	39	2.9
Translation. ribosomal structure and biogenesis	J	8	1.1	6	0.9	9	0.7
Transcription	K	**57**	**8.1**	**44**	**6.8**	82	6.1
Replication. recombination and repair	L	8	1.1	18	2.8	24	1.8
Cell wall/ membrane/ envelope biogenesis	M	32	4.6	28	4.3	46	3.4
Cell motility	N	4	0.6	9	1.4	47	3.5
Postranslational modification. protein turnover. chaperones	O	8	1.1	4	0.6	22	1.6
Inorganic ion transport and metabolism	P	43	6.1	30	4.6	61	4.6
Secondary metabolites biosynthesis. transport and catabolism	Q	13	1.8	15	2.3	23	1.7
Function unknow	S	344	48.9	381	58.8	731	54.6
Signal transduction mechanisms	T	34	4.8	5	0.8	**85**	**6.3**
Intracellular trafficking. secretion and vesicular transport	U	19	2.7	5	0.8	20	1.5
Defense mechanisms	V	7	1.0	11	1.7	14	1.0

Figure 26: Comparison of genomic diversity of *Pseudomonas* spp. strains. **(A)** Venn diagram in which each strain is represented by a circle. Overlapping regions show the number of coding sequences (CDS) conserved or not within the specified genomes. **(B)** List of the functional categories based on cluster of pan-genome orthologous groups of specific coding sequences (CDS) for each *Pseudomons* spp. strains. **(C)** Category percentage distribution of unique CDSs in each strain.
These results were generated using Ezbiocloud Comparative Genomics tools. n= number of CDSs detected for each category in each strain; %= percentage of each category from the total specific CDS detected for each strain; Grey color, categories with a higher number and percentage of CDSs for each *Pseudomonas* spp.; Pink arrow, specific categories overrepresented in each strain by comparison of category percentage with the other stains. PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P.chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110.

In other hand, *P. pseudoalcaligenes* AVO110 had the highest number of specific genes (n=1385), followed by *P. chlororaphis* PCL1601 with 722 specific genes and *P. chlororaphis* PCL1606 with 666 specific genes. Most of specific genes in each strains were related with production of hypothetical proteins with unknown function (PCL1606, 58.8%; AVO110, 54.6%; PCL1601, 48.9%). The rest of categories were distributed differently between strains. Both *P. chlororaphis* PCL1601 and PCL1606 showed a highest number and percentage of its specific genes related with transcription, whereas *P. pseudoalcaligenes* AVO110 showed a highest number and percentage of specific genes related with signal transduction mechanisms (e.g.: environmental responses regulation; Figure 26B-C).

Moreover, we compared the percentage of specific genes of each eggNOG category between strains and observed that PCL1601 presented a highest difference in percentage of specific genes related with amino acid metabolism (i.e.: amino acid ABC (ATP-binding cassette) transporter across cytoplasmic membrane) and intracellular trafficking categories (i.e.: Type I and III secretion systems). *Pseudomonas chlororaphis* PCL1606 showed a highest difference in percentage of specific genes related with replication-recombination category (e.g.: reparation or prevention of DNA damages). At last, *P. pseudoalcaligenes* AVO110 showed a highest differences in percentages in the number of specific genes related with cell motility (e.g.: flagellar synthesis; Figure 26B-C).

Due to the importance of production of secondary metabolites (Raaijmakers and Mazzola, 2012), both in community interaction and biological control activity, the putative secondary metabolites production were predicted using AntiSMASH software (Weber *et al.*, 2015). For this analysis, we selected the 50% of similarity as cutoff value to describe the cluster genes of secondary metabolites putative produced by our *Pseudomonas* spp. strains, predicted by comparison with AntiSMASH dataset and considering these results as an approximation of hypothetical metabolites production. In the genome of *P. chlororaphis* PCL1601 we found 5 cluster of genes with a percentage of similarity higher than 50% with described secondary metabolites clustered genes (n= 13, total number of cluster identified). They are involved in the putative production of pyoverdine, a non-ribosomal peptide synthase (NRPS) and other unidentified metabolites (Table 13). Moreover, this software detected the cluster gene involved in the production of phenazine (included in specific genes of this strain), but the percentage of similarity with other described phenazines was lower to 50% due to this cluster of genes presented a specific internal ABC-transporter. In *P. chlororaphis* PCL1606 genome we detected 12 cluster of genes, but only 5 of them with a similatrity higher than 50%, involved in the biosynthesis of pyoverdine, 2 antibiotics with antifungal activity, resorcinol and pyrrolnitrin (included in the specific genes of this strain); other NRPS and an unknown predicted protein with both of them a 100% of similarity with other *P. chlororaphis* strains; and a cluster of genes involved in the production of HSLs (homoserine lactones). Finally, for *P. pseudoalcaligenes* AVO110 genome, antiSMASH tool predicted only 2 cluster of genes with a percentage of similarity higher than 50%, one of them related with production of a polyketide synthase type I and other with the production of a NRPS (Table 13). The presence in this genome strain of other well-known antibiotics previously described for other *Pseudomonas* spp. was not detected.

Table 13: Predicted secondary metabolites produced by *Pseudomonas* spp. strains by using AntiSMASH software. **(A)** *Pseudomonas chlororaphis* PCL1601**. (B)** *Pseudomonas chlororaphis* PCL1606; **(C)** *Pseudomonas psedualcaligenes* AVO110.

A

***P. chlororaphis* PCL1601**		
	Putative product	%
Cluster1	ABC transporter-Phenazine*	35
Cluster4	Pyoverdine (NRPS)	97
Cluster8	Other	100
Cluster9	Pyoverdine (NRPS)	91
Cluster11	Other	60
Cluster12	NRPS	80

B

***P. chlororaphis* PCL1606**		
	Putative product	%
Cluster1	HSL	51
Cluster2	Pyoverdine (NRPS)	97
Cluster3	Resorcinol	91
Cluster5	Pyrrolnitrin	75
Cluster7	NRPS	100
Cluster12	Other	100

C

***P. pseudoalcaligenes* AVO110**		
	Putative product	%
Cluster5	NRPS	51
Cluster7	Type I PKS	58

%: percentage of similarity with described cluster of genes implicated in the synthesis of these products. *: cluster gene predicted with a similarity percentage less than 50%;

3.6 Analysis of volatiles compounds production

Recently, the role of volatile compounds in the biology of soil and rhizospheric bacteria have been reported (Garbeva *et al.*, 2014). For this, we focused in such VOCs production as an approach to start studying their role in the consortium mode of action.

Antagonistic activity

Dual-culture experiment using compartimentalized plates were performed to analyze the production of volatile organic compounds (VOCs) with antifungal activity against *R. necatrix* by *P. chlororaphis* PCL1601, *P. chlororaphis* PCL1606, *P. pseudoalcaligenes* AVO110 and the three *Pseudomonas* spp. inoculated together. Single *Pseudomonas* spp. strains tested were able to produce volatile compounds with antifungal ability, causing a significant reduction in fungal growth area comparing with control treatment. *Pseudomonas* spp. consortium produced VOCs that caused a fungal growth area reduction significantly different with single strains activity and control treatment (Figure 27).

Volatile organic compounds emission

Gas Chromatography-Mass Spectrophotometry (GC/MS)-based headspace analysis were performed to identify the volatile organic compounds produce by studied strains growing as single strains or as a microbial consortium. The spectrum of VOCs emitted contained many peaks but 20 of them (Table 14) were clearly and consistently identified in experimental samples relative to the control. *Pseudomonas chlororaphis* PCL1601 produce 5 VOCs, *P. chlororaphis* PCL1606 emit 8 VOCs (2 of them were specific for this strain) and *P. pseudoalcaligenes* AVO110 produce 13 VOCs (7 of them differently from those produced by the other two *P. chlororaphis* species). When 3 strains where co-inoculated, 15 volatile compounds where detected, displaying 1-undecene and dimethyl disulfide a shared production by three *Pseudomonas* spp. strains. In microbial consortium, one of the VOCs, the ketone 2-pentanone, was produced by PCL1601 whereas 6 of them were emitted by AVO110 (mercaptoacetone,

S-methyl propanethioate, S-methyl ester butanethioic acid, 5-undecene, 1-tridecene and 2-undecanone). Moreover, 3 of the microbial consortium VOCs were emitted only when three *Pseudomonas* spp. growth together such as hydrocarbons pentadecane and heptadecane and the ester S-methyl 3-methylbutanethioate. Furthermore, some VOCs as methyl thioacetate, methyl thiocyanate, 3-methyl butanenitrile, 2-methoxymethyl furan and 2-undecene were produced by at least some of the *Pseudomonas* spp. strains but not by the microbial consortium.

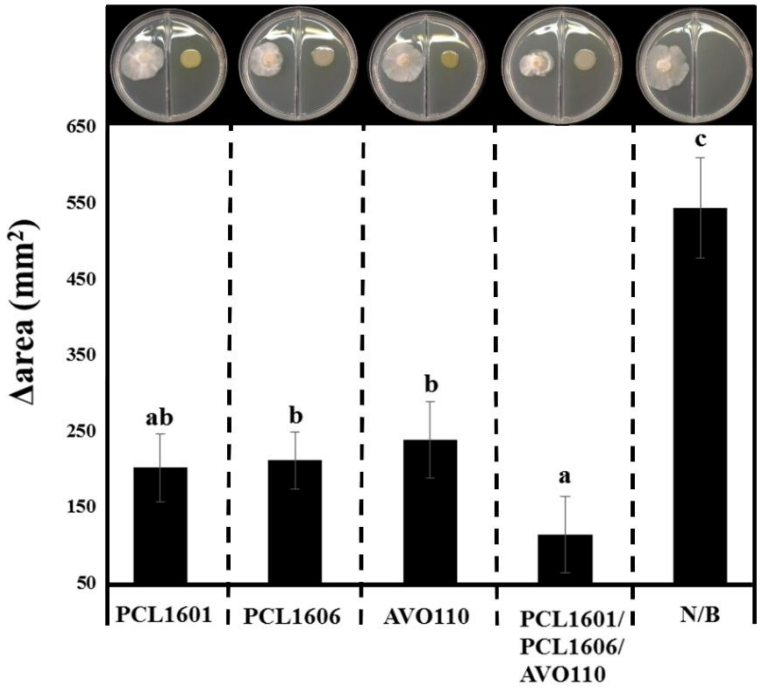


Figure 27: Inhibitory growth effect of *Rosellinia necatrix* caused by VOCs produced by *Pseudomonas* spp. strains. (Pictures) Dual plate assay using PDA/LB as media to growth *R. necatrix* and *Pseudomonas* spp. separately during 3 days. (Bar chart) Mycelial growth area of *R. necatrix* at 3 days since beginning of the experiment in presence of volatiles produced by *Pseudomonas* spp. and microbial consortium. Values are expressed as difference between *R. necatrix* growth area compared with the uninoculated plate (not inoculated with bacteria strains). Data were analyzed for significance using an arcsine square root transformation with analysis of variance followed by Fisher's least significant difference test ($p = 0.05$). Values with different letter indications denote a statistically significant difference. PCL1601: *Pseudomonas chlororaphis* PCL1601; PCL1606: *P.chlororaphis* PCL1606; AVO110: *P. pseudoalcaligenes* AVO110; 1601/1606/110: microbial consortium; N/B, negative control sterile LB medium.

Table 14: Volatiles organic compounds identified by gas chromatography/ spectrometry mass emitted to the headspace by *Pseudomonas* spp. strains and microbial consortium.

Volatile compound	RT (min)	KI	PCL 1601	PCL 1606	AVO 110	1601/ 1606/ 110
2-Pentanone	6.61	664				
Mercaptoacetone	6.89	678				
Methyl thioacetate	6.90	678				
Methyl thiocyanate	7.14	691				
3-Methyl butanenitrile	7.67	713				
Dimethyl disulfide	8.01	725				
S-methyl propanethioate	9.51	780				
2-methoxymethyl furan	10.35	809				
S-methyl ester butanethioic acid	11.13	833				
1-Nonene	12.41	872				
S-methyl 3-methylbutanethioate	14.16	924				
1-Decene	15.76	972				
1-Undecene	18.75	1074				
2-Undecene	19.04	1084				
5-Undecene	19.04	1084				
1-Dodecene	20.99	1169				
1-Tridecene	22.87	1265				
2-Undecanone	22.92	1268				
Pentadecane	25.98	1465				
Heptadecane	28.46	1661				

RT, retention time; KI, Kovats retention index; PCL1601, *Pseudomonas chlororaphis* PCL1601; PCL1606, *P.chlororaphis* PCL1606; AVO110, *P. pseudoalcaligenes* AVO110; 1601/1606/110, microbial consortium.

4. Discussion

Suppresiveness is a soil trait reflected by plant protection against some phytopathogens when growing in such soil (Weller *et al.*, 2002). The role of microbial communities in soil suppressiveness had been widely described along years (Lazarovits *et al.*, 2001; Bailey and Lazarovits 2003; van Elsas and Postma 2007; Bonilla *et al.*, 2012a). In all cases, two classical types of suppressiveness have been explained: total microbial community-dependent (general suppression) not transferable to other soil or specific group-dependent (specific suppression) transferable to other soil (Weller at al., 2002). In any case, the role of the microbiota is really important to control the disease index (Mendes *et al.*, 2011; Pane *et al.*, 2013; Bonilla *et al.*, 2015).

In the suppression of the avocado disease caused by *R. necatrix*, the increase in the relative abundance of some groups of microorganisms present in soils amended with composted almond shells was observed. Those groups included some representatives from bacterial classes *Gammaproteobacteria* and *Betaproteobacteria* (*Proteobacteria*) and fungal class *Dothideomycetes* (*Ascomycota*). Moreover, some of these representatives of the *Gammaproteobacteria* have been isolated in pure culture, and their biocontrol ability confirmed (Vida *et al.*, 2017a under review).

Regarding previous studies, *Gammaproteobacteria* is a class of *Proteobacteria*, widely described for their plant protection abilities and their fungal interactions in suppressive soils (Mendes *et al.*, 2011; Koyama *et al.*, 2014; Vida *et al.*, 2016). They include different fast-growing and easily cultivable bacteria. This is one of the reason because the isolation strategies of biocontrol agents (BCAs) against WRR from healthy avocado roots has been performed during last 10 years rendering potential biocontrol strains, mainly belonging to the genus *Pseudomonas* (Cazorla *et al.*, 2006; Pliego *et al.*, 2007, 2012; Gónzalez-Sánchez *et al.*, 2013). Three of the more efficient avocado BCAs already described and with a biocontrol ability are the strains of *Pseudomonas chlororaphis* PCL1601 (Cazorla *et al.*, 2006), *Pseudomonas*

chlororaphis PCL1606 (Cazorla *et al.*, 2006) and *Pseudomonas pseudoalcaligenes* AVO110 (Pliego *et al.*, 2007). The strains of *Pseudomonas chlororaphis* could perform their antagonistic activity by the production of antimicrobial compounds, such as proteases, lipases, siderophores and hydrogen cyanide, but mainly, by antifungal compounds such as phenazine-derivatives produced by PCL1601 and pyrrolnitin and 2-hexyl 5-propyl resorcinol induced by PCL1606 (Cazorla *et al.*, 2006; Calderón *et al.*, 2013). In other hand, *P. pseudoalcaligenes* AVO110 performed their biocontrol ability due to the competition for niche and nutrients (Pliego *et al.*, 2008).

Due to the different and complementary phenotypes of these bacteria, we have chosen them to design a microbial consortium in order to understand the bacterial interaction during biocontrol process. The construction of synthetic multicellular consortia have implications for understanding and manipulating natural communities and molecular mechanisms that stabilize social interactions (Teague *et al.*, 2015). Coexistence of multiple microorganims with similar trophic niches is regarded as one of the major factors to confer functional stability and resiliency on microbial ecosystems. In fact, construction of microbial model consortia, in which interspecies interactions in ecosystems are reproduced by defined co-culture of isolated microorganisms, is appreciated as a worthwhile method to investigate microbial interactions (Kato *et al.*, 2014).

Firstly, compatibility assays, among selected strains were performed, not showing an inhibition of plate growth among them, but with the strain used as control, *B. subtilis* PCL1608. This compatibility among *Pseudomonas* spp. but not with *Bacillus* spp. could be one of the basis for the low level of *Firmicutes* in the almond shells amended soil after increase of some specific groups of *Gammaproteobacteria* (Vida *et al.*, 2016).

The artificial consortium was colonizing abilities on avocado and wheat roots. Mixed cells forming a network that cover the root surface were observed when strains were

co-inoculated, suggesting a cell-to-cell interaction among them. Moreover, we observed a specificity of *Pseudomonas* spp. strains and the microbial consortium for avocado roots (independently of the presence or absence of *R. necatrix).* This phenotype can be explained as a bacterial response to specific compounds present in root exudates and their niche specialization where they were isolated from (Barret *et al.*, 2011).

Due to its compatibility and different and/or putative complementary mode of action in avocado rhizosphere, we confirmed that this artificial microbial consortium retained the biocontrol activity. We cannot observed significant differences in reduction of disease index when we used wheat-*R. necatrix* system. However, in avocado-*R.necatrix* system experiments, we observed significant differences in disease index between control and bacterized plants. By inoculating avocado root with PCL1601, PCL1606 and AVO110 together (in ratio 1:1:1), biocontrol activity against *R. necatrix* had no significative differences with the biocontrol displayed by individual strains. These results also support the possible specificity of these strains to avocado roots.

The comparative analysis of genomes performed in this study help us to understand the potential microbial interactions that could occur during biocontrol process between the bacterial partners of the consortium PCL1601, PCL1606 and AVO110.

Analysis of distribution of eggNOG categories (hierarchical clusters of orthologous groups) in each bacterial genome showed the ability of these strains to coexist in a similar environment due to the high similarity between orthologous groups of genes, and only observed subtle differences in some of the categories (Silby *et al.*, 2011). A comparative analysis of clusters of orthologous groups between genomes (Pan-genome orthologous genes, POGs) showed that 36% of genes where shared by studied Pseudomonads, forming the gene core of these strains and probably related with their ability to inhabit in the same ecological niche, the avocado rhizosphere. This core of genes are formed by genes with an assigned putative function related with general

metabolism (carbohydrate, amino acid, lipid, etc) (Silby *et al.*, 2011; Calderón *et al.*, 2015).

Nevertheless, differences between strains were detected, reflecting its potential specialization activities. The comparison of POGs categories help us to detect the specific genes for each *Pseudomonas* spp. species, complemented by the prediction of putative secondary metabolites production were performed using the online tool AntiSMASH. This analysis revealed that *Pseudomonas pseudoalcaligenes* AVO110 presents the highest percentage of specific genes, probably because this strain is a different specie in the microbial consortium, also with a different mode of action against *R. necatrix*. Some of AVO110 specific genes were related with cell motility, concretely with flagellar synthesis and chemotaxis, showing the importance for this strain of a directed motility and supporting the importance for this bacterium to efficiently colonize avocado roots (de Weert *et al.*, 2002; Pliego *et al.*, 2007). No genes of production of putative antifungal compounds were detected in the genome analysis of *P. pseudoalcaligenes* AVO110. On the other hand, specific genes of *P. chlororaphis* PCL1601 showed a highest percentage in categories related with amino acid metabolisms and intracellular trafficking of compounds, suggesting the importance of environmental communication. Moreover, *P. chlororaphis* PCL1601 is able to produce phenazines (Cazorla *et al.*, 2006), antibiotics described in different studies for their impact on the behavior of bacteria in the environment, since it could act as cell signals that regulate patterns of gene expression, contribute to biofilm formation and architecture, and enhance bacterial survival (Pierson and Pierson, 2010). The analysis of PCL1601 genome showed a cluster of genes involved in the production of pyoverdine and an unknown compound as well as, phenazine associated with an ABC transport, suggesting the directed and controlled secretion of this antimicrobial compounds to the environment (Dietrich *et al.*, 2006; Selin *et al.*, 2011).

Finally, the specific genes found in *P. chlororaphis* PCL1606 genome are involved in DNA replication and recombination. Concretely, we found specific genes related with reparation or prevention of DNA damages, suggesting a high capacity of environmental adaptation due to bacterial damage repair mechanisms maintain the integrity of genomes as well as have broader roles, including responses to stress, long-term colonization and virulence (Zgur-Bertok, 2013). Moreover, *P. chlororaphis* PCL1606 could produce pyoverdine and antibiotics pyrrolnitrin (PRN) and 2-hexyl 5-methylresorcinol (HPR), checked in previous studies (Cazorla *et al.*, 2006; Calderon *et al.* 2015). Additionally, production of some homoserine lactones (HSLs), were detected using *C. violaceum* CV026 as biosensor (McClean *et al.*, 1997), as well as, the genes related with their production, but not present in PCL1601 and AVO110. The HSLs production was maintained in microbial consortium, showing that a *quorum sensing* regulation via exogenus HSLs could take place and, *quorum quenching* mechanisms could be avoided in this artificial consortium (Grandclément *et al.*, 2016). Different studies showed the importance of cell-to-cell communication inside the microbial community using different acyl-homoserine lactones in Gram-negative bacteria (Parsed and Greenberg, 2000). This hypothesis would need further experimentation in order to analize the production of these molecules involved in the intra- and interspecies relationships regulated via *quorum sensing*.

Finally, as first approach to study cells interactions, the production of volatile organic compouns (VOCs) have been studied. Several works demonstrated that bacterial volatiles could have many functions, among other, such as communication signals and growth-promoting or inhibiting agents (Effmert *et al.*, 2012). They are important for the sustainment of bacterial populations in ecological niches and for the cooperative development of a community of different organisms because volatiles not only play a role above ground but also function below ground due to their ability to diffuse through aqueous solutions (Kai *et al.*, 2009). For this reasons, some volatiles could have

antimicrobial activity and may play a significant role in long-distance interactions among soil microorganisms, thus contributing to the activity of suppressive soils (Raaijmaker and Mazzola, 2012). The analysis of VOCs production resulted in many different volatile compounds, but few of them directly related to Pseudomonas-produced VOCs with biocontrol activity (Effmert *et al.*, 2012). Thus, dimethyl disulfide (DMDS) and 1-undece volatiles, both were produced in all conditions assayed and described for their antagonistic activity in previous works (Fernando *et al.*, 2005; Popova *et al.*, 2014; Hunziker *et al.*, 2015). Another of these VOC *Pseudomonas*-produced was S-methyl butanethioate, volatile compounds which seem to have a specific effect on inhibition of sporangia germination, mycelial growth and zoospore motility of oomycete (de Vrieze *et al.*, 2015). A similar compounds, S-methyl 3-methylbutanethioate (MMBT) were emitted by the bacterial consortium, assigning a putative role in the inhibition effect of *R. necatrix*. Nowadays, nothing is known about bacterial metabolic pathway for this compound, so the putative production by a metabolism based in the interaction of the *Pseudomonas* spp. strains should need further experimentation.

In the same way, two volatiles hydrocarbons, pentadecane and heptadecane, were produced just by the bacterial consortium. These compounds are alkanes, molecules produced directly from degradation of fatty acids and implicated in different issues such as plant cuticular waxes, insect pheromones and mainly with unknown functions in other numerous organisms (Schrimer *et al.*, 2010). Actually, the production and function of these volatiles are not well understood, so we only can suggest a hypothetical co-metabolism for its biosynthesis.

5. Conclusion

In this study, we have focused in the modelling of an artificial bacterial consortium based in compatible *Pseudomonas* spp. strains and with biocontrol ability against *R. necatrix*. We observed that these strains are able to colonize roots forming mixed cells networks where interaction patterns could occur. These mixed networks are stable in avocado roots and could increase the plant resistance to a wide range of stress, as the pathogen challenge. Moreover, from a genotypic point of view, these strains could cooperate among them at same time that could perform different specific roles in the niche that they inhabit. As first step, we started the analysis of VOCs production and their implications in the antifungal activity.

Future transcriptomic experiments, directed to analyze the RNA sequences from microbial consortium interactions on avocado roots will be carried out to better understand community interactions during biocontrol process.

CHAPTER V

GENERAL DISCUSSION

Environmental and food quality concerns have increased in the current society, causing a generalized interest for sustainable agriculture. Environmentally safe farming procedures include the reduction of the use of agrochemical products, in favour of other type of techniques less aggressive, as the use of tolerant rootsocks to abiotic and biotic stress, as well as the maintenance of soil quality to improve plant health and productivity (Doran and Parkin, 1994). In the case of the control of soilborne pathogens, different agricultural practices compatible with organic farming have been applied, including crop rotation (Bailey and Lazarovits, 2003), minimal tillage practices (Bailey and Lazarovits, 2003), soil solarisation (López-Herrera *et al.*, 1998), the application of single biocontrol agents (Cazorla *et al.*, 2006, 2007; Calderón *et al.*, 2013), and the topic discussed in this stuy, the application of organic amendments (Bailey and Lazarovits, 2003).

The quantity and quality of organic matter input affect physicochemical properties of the soil but also could modify biotic factors related to the soil microbiota, such as microbial biomass and diversity, community structure and soil activities with a direct effect in biological control of diseases (Hadar and Papadopoulou, 2012). In this sense, the application of different types of organic amendments as suppressive compost, could provide an environment in which plant disease development is reduced, even in the presence of a pathogen and a susceptible host (Hadar and Papadopoulou, 2012). This effect is related with the microbial communities evolved after the amendment, which play a crucial role in the functioning of plants by influencing their physiology and development (Mendes *et al.*, 2013) and effectively protecting them against soilborne pathogens (Weller *et al.*, 2002). From these experiences, two classical types of suppressiveness have been explained: total microbial community-dependent (general suppression) not transferable to other soil or specific group-dependent (specific suppression) transferable to other soil (Weller at al., 2002).

Our model of study is the avocado, which is a significant crop in southern of Spain, the main exporter of avocado fruit to European Union (ASAJA Málaga http://www.asajamalaga.com/?n=1596). Climate conditions of this area have promoted the cultivation of this subtropical tree, but local farmer have had to face different cultivation problems, including a low amount of organic matter in the soils and the emergence of soilborne fungal pathogens, such as *Rosellinia necatrix* that causes avocado white root rot (WRR; Perez-Jimenez, 2008).

For several years, organic amendment have been performed by farmers due to the key role that the organic matter play in long-term sustainability of tropical and subtropical orchards and in maintenance of soil quality (Doran and Parkin, 1994). Previous studies showed the beneficial effects of the application of organic amendment in avocado crops, increasing feeder roots growth and overall plant health, reducing plant stress and increasing yield (Wolstenholme *et al.*, 1997). The use of an appropriate organic amendment is a key tool in organic management of most woody perennial crops. Moreover, and from a sustainable point of view, the best option to select an organic amendment should be a residual organic material from surrounding agro-industrial activities. After some treatments (such as composting), this organic matter can be reuse safely, helping to reduce the amonts of residues and improving some characteristics of the agricultural soil (Hadar and Papadopoulou, 2012). In southern of Spain, almond shells were an easily available residue rich in lignin, long-lasting, with low cost and resistance against wind, becoming in a highly recommended mulch (López *et al.*, 2014). In the case of avocado crop, previous studies showed that the application of composted almond shells as organic amendment induced subtle changes in bacterial community composition and specific enzymatic activities, related to the direct inhibition of *R. necatrix* by this amendment (Bonilla *et al.*, 2015). Several examples have described this kind of induced suppressive phenotype against different plant pathogen by the application of a wide range of mulches (Mendes *et al.*, 2011; Pane *et*

al., 2013). In this study, physicochemical changes that take place in the avocado plant and soil after the application of composted almond shells was evaluated. Amended soil with composted almond shells, showed a high value of C/N ratio, result of the slow degradation rate of this organic input, which could allow the accumulation of different C sources available for plants and microorganisms. Furthermore, we observed an increase in micronutrients such as Fe, Mn, Cu and Zn that could cause a positive effect both in plant health as in soil microbiome function (Gupta *et al.*, 208). The reduced rate of organic matter mineralization was sufficient in previous studies to supply the needed nutrients ant to maintain or even increase fruit yield in an almond shells amended soil (López *et al.*, 2014).

In order to evaluate the effect of the application of composted almond shells in avocado crops, we performed assays that confirmed the induction of suppressiveness against *R. necatrix* in the amended soils. Moreover, the suppressive soil phenotype was directely related with the activity of the soil microbial community, evolved in that environment modified with the addition of this organic matter. Microbial-based suppressiveness was related due to the reduction of population density by a moist-heat treatment, causing a decreased in the suppressiveness. Furthermore, suppressive ability was recovered by complemented soils, showing that a microbial-specific suppressiveness was occurring in this amended soil as previously described in several studies (Weller *et al.*, 2002; Mendes *et al.*, 2011; Pane *et al.*, 2013; Bonilla *et al.*, 2015).

To decipher the key role of the microbial community in the induction of suppressiveness in this avocado crop soil, the analysis of the prokaryotic and eukaryotic profiles developed were necessary. Historically, different methods have been used to characterize the microbial composition of a soil community, including the use of selective media and plate counts assays and more recently, the use of molecular techniques based on the characterization of soil-extracted DNA (Jeewon and Hyde, 2007). Specifically, the direct sequencing of the 16S rRNA gene and ITS

regions of amended soil DNA, showed an increase in the relative abundance of some groups of microorganisms with potential antagonistic activities against *R. necatrix*. In general, a specific increase of *Gammaproteobacteria* and *Dothideomycetes* was observed, but also, a clear reduction in the fungal group of *Xylariales*, where *R. necatrix* is allocated (Vida *et al.*, 2016) thus evidencing the suppressive effect. The obtained results predicted a putative sequential activity of fungal and bacterial specific groups, which could finally allow the inhibition of *R. necatrix* growth. These results suggest an implication of members of fungal order *Pleosporales* in the direct degradation of lignin (Ortíz-Bermúdez *et al.*, 2007), that could result in an increase in C sources more available for bacterial members of classes *Gamma-* and *Betaproteobacteria*, with a faster growth, with some members that could display activities against plant pathogenic fungi (Postma *et al.*, 2010), and with a demonstrated capacity to assimilate and/or degrade aromatic and toxic compounds present in plant exudates and soils, allowing them to acquire a selective advantage (Barret *et al.*, 2011). Additionally, the putative functional profile of DNA extracted from amended soils was obtained by microarray hybridization approaches (GeoChip 4.6®) and allowed us to consider the microbial community from a functional point of view and to determine overrepresented activities that could be possibly related with the suppressive effect. Thus, the functional profile obtained showed the presence of antimicrobial production genes overrepresented in amended soil associated with different genera of *Gamma-* and *Betaproteobacteria*, previously described as biological control agents against different plant pathogen (Raaijmakers and Mazzola, 2012). This is in agree with the prokaryotic profile observed showing the increase in the relative abundance of *Pseudomonas* spp. strains and related groups, suggesting that they could be involved in the induction of suppressiveness against *R. necatrix* in amended soils with composted almond shells. Several previous biological control studies have focused on

the behaviour and mechanisms as beneficial biocontrol organisms of members from this *Pseudomonas* spp. group (Weller *et al.*, 2002).

Since our results targeted some of the families of *Gammaproteobacteria* possibly involved in the induction of soil suppressiveness against avocado white root rot, we further performed the isolation of *Pseudomonas* spp. and other groups, from composted almond shells amended soils in order to confirm their suppressive phenotype against *R. necatrix*. A collection of 246 isolates was obtained and characterized. The collection was composed mainly by putative Enterobacteria, Xanthomonads and Pseudomonads isolates with a wide range of activities involved in biological control as putative production of antifungal compounds or production of lytic exoenzymes and plant growth promotion related activities. Several mechanisms used by bacteria have been described as effective in biological control, indirectly by causing plant growth promotion as solubilization of phosphate (Lugtenberg and Kamilova, 2009) or directly, as the production of antimicrobial metabolites that constitute an important element of the complex adaptive capabilities that enable microorganisms to respond appropriately to their neighbors, including soilborne pathogens (Raaijmakers and Mazzola, 2012).

In this study, some diverse representatives of genera *Pseudomonas* sp., *Serratia* sp., and *Stenotrophomonas* sp., have been isolated and selected to finally perform biological control assays against *R. necatrix*, showing all of the tested isolates (n=8) biological control activity in different level. This results evidence that *Pseudomonas* spp. and related groups strains, evolved into an amended soil with composted almond shells, could be involved in the suppressiveness-induced against *R. necatrix*. Bacterial species belonging to the genera *Bacillus*, *Burkholderia*, *Collimonas*, *Serratia* and *Pseudomonas* have been widely studied from a biocontrol perspective and found to use a wide range of mechanisms influencing plant growth and health (Mendes *et al.*, 2013). Recent works described *Serratia* spp. strains as plant-associated bacteria

screened for their antagonistic activity against soilborne pathogens (Berg, 2000; Beneduzi *et al.*, 2013; Gkarmiri *et al.*, 2015), as well as, *Pseudomonas* spp. strains with fungal biocontrol activity by production of antimicrobial compounds (Deng *et al.*, 2015; Kamou *et al.*, 2016). *Stenotrophomonas* spp. strains have been described in literature by its ability to both promote plant growth and protect roots against biotic and abiotic stress (Alavi *et al.*, 2013). Our results are in agree with the presence of such genera in literature. Its broad presence could be a result of easy and fast growth on artificial media of these common inhabitants of soil and plant rhizosphere and producers of different secondary metabolites (Raaijmakers and Mazzola, 2012). Moreover, some studies described that the presence of determined saprophytic fungi in soil and rizhosphere environment can be an additional factor that influence the soil bacterial community (de Boer *et al.*, 2015). In this sense, in suppressiveness-induced soil amended with composted almond shells, the presence of fungal species of *Pleosporales*, with a reported saprophytic activity degrading lignin compounds (Ortíz-Bermúdez *et al.*, 2007) from almond shells, could favour the selection among others, of fast-growing *Pseudomonas* spp., *Serratia* spp. and *Stenotrophomonas* spp. with antagonistic abilities.

Especial interest had the genus *Pseudomonas* sp., which is a group of microorganisms worldwide described and containing many representatives considered as biological control agents against different plant diseases, including avocado white root rot (Cazorla *et al.*, 2006). Three of the more efficient Pseudomonads previously isolated and with biological control against *R. necatrix*, were selected to design an artificial microbial consortium. This synthetic community would be used in order to understand the multitrophic interactions that could be occurring during suppressive activity against *R. necatrix*. In recent years, a promising way to overcome the difficulties associated with studying natural communities is to create artificial communities that retain the key features of their natural representatives (Großkopf and Soyer, 2014). In

this study, we selected three well-known avocado biocontrol agents, *Pseudomonas chlororaphis* PCL1601 and PCL1606, and *Pseudomonas pseudoalcaligenes* AVO110, due to they have showed different features involved in their biocontrol activities (Cazorla *et al.*, 2006, Pliego *et al.*, 2007). *Pseudomonas chlororaphis* strains secreted different hydrolytic enzymes and can produced hydrogen cyanide as well as other types of antimicrobial compounds as phenazines (PCL1601), pyrrolnytrin and 2-hexyl 5-propyl resorcinol (PCL1606), all related with their antifungal activity (Cazorla *et al.*, 2006). However, *P. pseudoalcaligenes* AVO110 did not produced any antimicrobial compounds tested, but showed a strong ability to colonize avocado roots due to their ability to detect avocado exudates (Pliego *et al.*, 2007). The combination of those rhizobacteria with different modes of action would be an easy way to study their interactions during the multitrophic interactions that take place in the biocontrol activity.

Before further experiments, we evaluated the compatible interaction between them by plate assays. The result showed a compatible plate growth among the three Pseudomonads used to form the microbial consortium, but not with Gram-positive rhizobacteria *Bacillus subtilis* PCL1608. This incompatibility between *Gammaproteobacteria* and *Firmicutes* could be one of the basis of the low level of *Bacillus* spp. and other Gram-positive strains in the amended soils (Vida *et al.*, 2016). Additionally, the microscopy visualization assays showed stability of the artificial consortium on avocado roots. An organization of mixed cell of the three partners of the consortium was observed under microscopy. This characteristic root colonization pattern of the microbial consortium forming mixed cells networks with short distance between cells of the different strains, could favor their communication. Previous studies described that spatial organization and architecture of microbial communities is crucial to maintain a stable community (De Roy *et al.*, 2014), showing a functional link between species clustered together in a microbial community (Daims *et al.*, 2006)

or balancing the competition and beneficial interactions in the stable community (Kim *et al.*, 2008). In this point, we confirmed by performing biological control assays against *R. necatrix* the maintenance of the biocontrol phenotype by the microbial consortium. Moreover, these approaches could suggest that the biological control process would be occurring as an integrated activity similar to the suppressive community could behave in the natural environment. Different studies showed that the species interactions into microbial communities can be either metabolism-based or be driven by social traits. Specially, social interactions between strains and species is crucial to understand microbes and how they respond to perturbations (Großkopf and Soyer, 2014).

Previous studies, suggested that the genotypic view of microbial interactions could help to gain insight in the analysis of the cooperative or competitive behaviour of a microbial community, suggesting that cells of the same genotype will cooperate, whereas different genotypes will typically compete (Mitri and Foster, 2013). For this reason, we performed a genome-wide analysis of the three selected Pseudomonads in order to know their putative similarities and differences and understand their putative interactions. The comparative genome analysis performed showed that three strains shared a high percentage of genes, due to their taxonomical relation (all of them belong to the genus *Pseudomonas* spp.) and related to their ability to inhabit in the same ecological niche (all of them isolated from avocado roots) similar to other examples previously reported (Loper *et al.*, 2012). Nevertheless, each of the strains showed groups of specific genes suggesting different specialization inside the same niche: *P. pseudoalcaligenes* AVO110 in the efficient colonization of avocado roots and perceiving avocado exudates, *P. chlororaphis* PCL1601 in the putative environmental exchange of compounds and *P. chlororaphis* PCL1606 in the adaptation to different environmental conditions and both of them with strong antifungal activity. All these results, supports the different putative mode of action of these strains in the avocado

rhizosphere, but as a putative cooperative community due to a high relatedness within strains and a not overlapping niche, probably favoring the microbial cooperation, as previously proposed (Mitri and Foster, 2013).

As a first step to understand the interactions that take place inside the microbial consortium, we performed an *in silico* analysis of the putative production of secondary metabolites (Weber *et al.*, 2015). Numerous studies have demonstrated that metabolites, including antibiotics, enzymes and volatiles produced by soil- and plant-associated bacteria, are key factors in the suppression of plant pathogens (Gross and Loper, 2009; Raaijmakers and Mazzola, 2012). In this case, differential patterns of secondary metabolites production were observed for each strain, supporting the results obtained in previous studies (Cazorla *et al.*, 2006; Pliego *et al.*, 2008).

Futhermore, the *in vitro* analysis of production of secondary metabolites were extended to volatiles organic compounds (VOCs). Volatiles molecules can act over a wider range of scale than non-volatiles as they can diffuse through both the liquid and gaseous phases of the soil (Effmert *et al.*, 2012). In this study, we demonstrated the antifungal activity of the microbial consortium against *R. necatrix* mediated by VOCs production. For this reason, we analyzed the VOCs emitted and described by its fungal growth inhibitory effect, such as dimethyl disulfide (DMDS) and 1-undecene (Popova *et al.*, 2014). These compounds were produced by single strains and microbial consortium and could be involved in their antagonistic activity. Nevertheless, the antifungal activity of microbial consortium was higher than activities of singles strains so, the bacterial community should be able to efflux other compounds implicated in the antagonistic phenothype. One of these volatiles could be S-methyl 3-methylbutanethioate (MMBT), emitted only by microbial consortium and very similar to a previously described compound, S-methyl butanethioate, volatile compounds which have a specific effect on inhibition of sporangia germination, mycelial growth and zoospore motility of oomycete (de Vrieze *et al.*, 2015). Nevertheless, the bacterial

metabolic production pathway of this VOC is unknown, so the posibility of the ocurrence of a metabolism-based in the interactions of the bacterial consortium could be only suggested.

Moreover, another two metabolites are also produced in microbial consortium. These compounds are pentadecane and heptadecane, alkanes derived from long-chain fatty acid degradation (Schrimer *et al.*, 2010). These types of compounds have been described as component of waxes, concretely of plant cuticular waxes, products with a crucial importance in protecting plant surfaces and in bacteria waxes, products with a potential use as carbon and energy storage compound (Ishige *et al.*, 2003). In the same way, that previous VOCs compound, a putative metabolism-based in the bacterial interactions could performed the synthesis of these compounds to be used as energy storage and even as a protection layer by the bacterial consortium in order to improve their establishment in the rhizosphere, but its co-metabolic production should be tested in future experiments.

In this point, we proposed the artificial bacterial consortium form by *P. chlororaphis* PCL1601, PCL1606 and *P. pseudoalcaligenes* AVO110 as a compatible and stable model of bacterial community with biological control activity against the avocado soilborne pathogen *R. necatrix*. Our purpose is performing future experiments (transcriptomic, proteomic, metabolomic) and deeping in the knowledge of the multitrophic interactions occurring during biocontrol in avocado roots, in order to understand the microbial ecology of suppressiveness-induced agricultural soil by the application of composted almond shells.

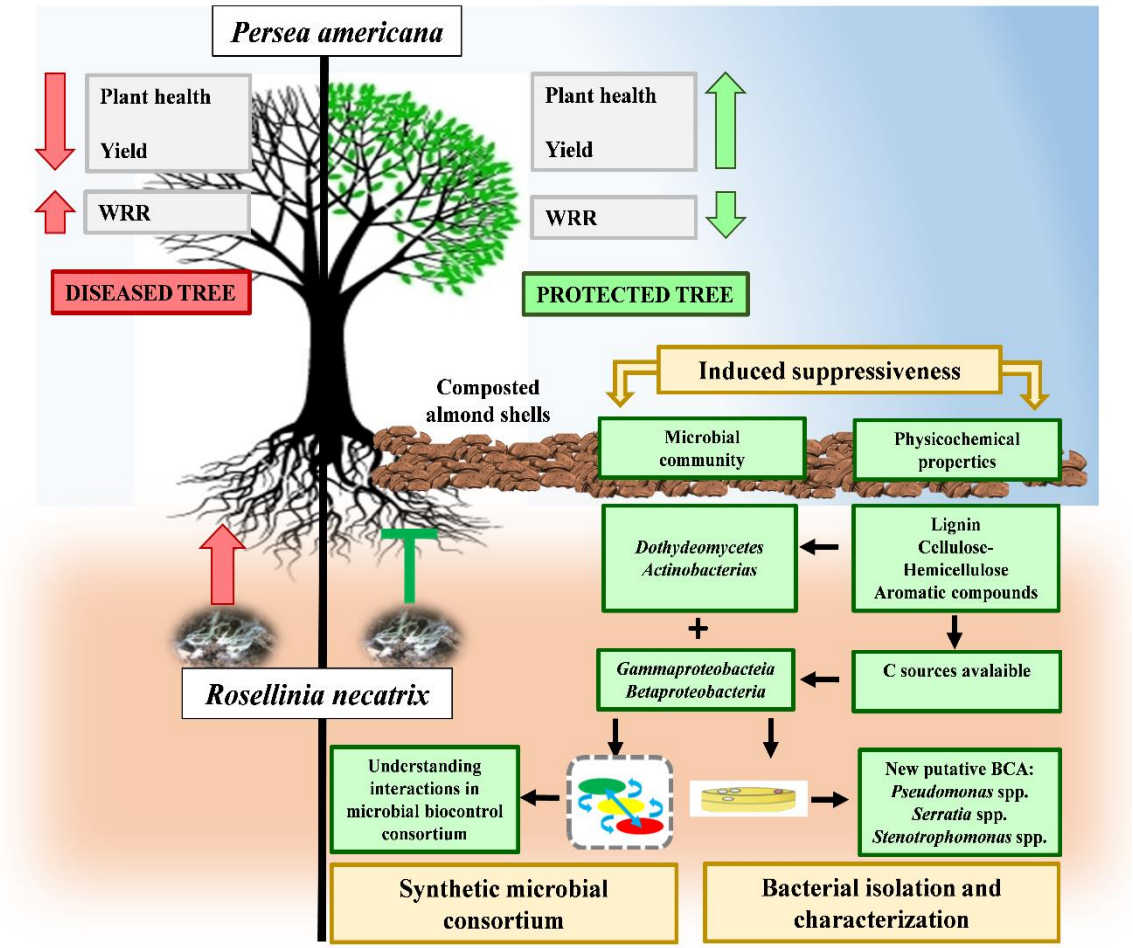


Figure 28: Soil suppressiveness against *Rosellinia necatrix* due to the effect of composted almond shells amendmen, on avocado crops. WRR, avocado white root rot.

In Figure 28, we summarized the mains finding of this work. The application of composted almond shells onto an avocado crop soil, induced changes in its physicochemical and microbial properties. The microbial community developed, induced suppressiveness against *Rosellinia necatrix*, due to the sequential activities of specific groups of microorganism, such as fungal members of class *Dothydeomycetes*, putative involved in the degradation of composted almond shells. Bacterial members of classes *Gamma-* and *Betaproteobacteria*, could take advantage by their ability to use the C source available, and increased their relative abundance. The antifungal activities of many of bacterial representatives of these groups could decrease the relative abundance of *R. necatrix* in this soil. The isolation of bacterial strains from *Gammaproteobacteria* class, particularly from *Pseudomonas* sp. genus from the amended soil, have shown the potential biological control agents against this avocado soilborne pathogen. Taking three biocontrol isolated from avocado soil and rhizosphere, allowed the modelling of an artificial bacterial consortium that retained the biological control activity agains *R. necatrix.* This bacterial consortium showed compatibility during *in vitro* and *in vivo* assays, and will help us in the knowledge of the multitrophic interactions that take place in a suppressive soil during the biocontrol activity.

CONCLUSIONS

1.- The microbial community developed in an avocado field soil, amended with composted almond shells, is responsible of the induction of suppressiveness against *Rosellinia necatrix*.

2.- The metagenomics analysis showed the relevance in this suppressive soils of some specific groups of microorganisms, such as fungal members of class *Dothydeomycetes* and bacterial members of class *Gammaproteobacteria*.

3.- New biological control agents from *Gammaproteobacteria* class, particularly from *Pseudomonas* sp., *Serratia* sp. and *Stenotrophomonas* sp. genus, against *Rosellinia necatrix* have been isolated from this amended suppressive soil.

4.-We have developed a compatible artificial bacterial consortium form by *Pseudomonas chlororaphis* PCL1601, *Pseudomonas chlororaphis* PCL1606 and *Pseudomonas pseudoalcaligenes* AVO110, that showed a mixed and stable colonization root pattern. This artificial consortium retained the biological control activity against *Rosellinia necatrix* in avocado roots, and was able to produce volatiles organic compounds with antifungal activity.

CONCLUSIONES

1.- La comunidad microbiana que se desarrolla en un suelo de cultivo de aguacate, enmendado con cáscara de almendra, es responsable de la inducción de supresividad frente a *Roselinia necatrix.*

2.- El análisis metagenómico, pone de manifiesto la relevancia en estos suelos supresivos de determinados grupos de microorganismos, como la clase fúngica *Dotideomicetes* y de la clase bacteriana *Gammaproteobacteria.*

3.- Se han aislado nuevas cepas de gammaproteobacterias, en particular de los géneros *Pseudomonas* sp., *Serratia* sp. y *Stenotrophomonas* sp., con actividad de control biológico frente a *Rosellinia necatrix* desde estos suelos supresivos enmendados.

4.- Se ha desarrollado un consorcio bacteriano artificial compatible formado por *Pseudomonas chlororaphis* PCL1601, *Pseudomonas chlororaphis* PCL1606 y *Pseudomonas pseudoalcaligenes* AVO110 que muestra un patrón de colonización de raíz mixto y estable. Este consorcio mantiene la actividad de control biológico frente a *Rosellinia necatrix* en la raíz de aguacate, y además, tiene capacidad para producir compuestos orgánicos volátiles con actividad antifúngica.

REFERENCES

Abd-Alla, A., Jousset, F.X., Li, Y., Fédière, G., Cousserans, F. and Bergoin, M. (2004). NS-3 protein of the *Junonia coenia* densovirus is essential for viral DNA replication in an Ld652 cell line and *Spodoptera littoralis* larvae. *J. Virol.* 78, 790-797. doi: 10.1128/JVI.78.2.790-797.2004.

Agrios, G.N. Ed. (1997). *Plant Pathology.* Academic Press, San Diego, CA.

Akhter, A., Hage-Ahmed, K., Soja, G. and Steinkellner, S. (2015). Compost and biochar alter mycorrhization, tomato root exudation, and development of *Fusarium oxysporum* f. sp. *lycopersici*. *Front. Plant Sci.* 6:529. doi: 10.3389/fpls.2015.00529.

Alavi, P., Starcher, M., Zachow, C., Müller, H. and Berg, G. (2013). Root-microbe systems: the effect and mode of interaction of stress protecting agent (SPS) *Stenotrophomonas rhizophila* DSM14405^{T}. *Front. Plant Sci.* 4:141. doi: 10.3389/fpls.2013.00141.

Alabouvette, C., Olivain, C. and Steinberg, C. (2006). Biological control of plant diseases: the European situation. *Eur. J. Plant Pathol*. 114, 329–341. doi: 10.1007/s10658-005-0233-0.

Almario, J., Muller, D., Défago, G. and Moënne-Loccoz, Y. (2014). Rhizosphere ecology and phytoprotection in soils naturally suppressive to *Thielaviopsis* black root rot of tobacco. *Environ. Microbiol.* 16, 1949-1960. doi: 10.1111/1462-2920.12459.

Aryantha, I.P., Cross, R. and Guest, D.I. (2000). Suppression of *Phytophthora cinnamomi* in potting mixes amended with uncomposted and composted animal manures. *Phytopathology* 90, 775–782. doi:10.1094/PHYTO.2000.90.7.775.

Bai, S., Li, J., He, Z., Van Nostrand, J.D., Tian, Y., Lin, G. *et al.* (2013). GeoChip-based analysis of the functional gene diversity and metabolic potential of soil microbial communities of mangroves. *Appl. Microbiol. Biotechnol*. 97, 15:7035-7048. doi:10.1007/s00253-012-4496-z.

Bailey, K.L. and Lazarovits, G. (2003). Suppressing soil-borne diseases with residue management and organic amendments. *Soil Tillage Res*. 72, 169–180. doi:10.1016/S0167-1987(03)00086-2.

Bakker, P.A.H. M., Doornbos, R.F., Zamioudis, C., Berendsen, R.L. and Pieterse, C.M. J. (2013). Induced systemic resistance and the rhizosphere microbiome. *Plant Pathol. J.* 29, 136–143. doi:10.5423/PPJ.SI.07.2012.0111.

Bankhead, S.B., Thomashow, L.S. and Weller, D.M. (2016). Rhizosphere competence of wild-type and genetically engineered *Pseudomonas brassicacearum* is affected by the crop species. *Phytopathology* 106, 6: 554-561. doi: 10.1094/PHYTO-09-15-0244-R.

Barahona, E., Navazo, A., Yousef-Coronado, F., Aguirre de Cárcer, D., Martínez-Granero, F. *et al.* (2010). Efficient rhizosphere colonization by *Pseudomonas fluorescens* f113 mutants unable to form biofilms on abiotic surfaces. *Environ. Microbiol*. 12, 3185-3195. doi: 10.1111/j.1462-2920.2010.02291.x.

Bardou, P., Mariette, J., Escudié, F., Djemiel, C. and Klopp, C. (2014). Jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics* 15: 293. doi: 10.1186/1471-2015-15-293.

Barret, M., Morrissey, J.P. and O'Gara, F. (2011). Functional genomics analysis of plant growth–promoting rhizobacterial traits involved in rhizosphere competence. *Biol. Fert. Soils*. 47, 729–743. doi:10.1007/s00374-011-0605-x.

Bashan, Y. and Holguin, G. (1998). Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-pgpb (plant growth-promoting bacteria) and pgpb. *Soil Biol. Biochem.* 30: 819, 1225-1228.

Bender, G.S., Casale, W.L. and Rahimian, M. (1992). "Use of worm-composted sludge as a soil amendment for avocados in *Phytophthora*-infested soil" in *Proceeding of Second World Avocado Congress*. (Orange, CA), 143.

Beneduzi, A., Moreira, F., Costa, P.B., Vargas, L.K., Lisboa, B.B., Favreto, R., Baldani, J.I. and Passaglia, L.M.P. (2013). Diversity and plant growth promoting evaluation abilities of bacteria isolates from sugarcane cultivated in the south of Brazil. *Appl. Soil Ecol*. 63, 94-104. doi: 10.1016/j.apsoil.2012.08.010.

Berg, G. (2000). Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *J. Appl. Microbiol.* 88, 952–960. doi:10.1046/j.1365-2672.2000.01064.x.

Berg, G. and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13. doi:10.1111/j.1574-6941.2009.00654.x.

Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014). The plant microbiome and its importance for plant and human health. *Front. Microbiol*. 5:491. doi: 10.3389/fmicb.2014.0049.

Berg, G., Krause, R. and Mendes, R. (2015). Cross-kingdom similarities in microbiome ecology and biocontrol of pathogens. *Front. Microbiol*. 6: 1311. doi: 10.3389/fmicb.2015.01311.

Bertrand, H., Nalin, R., Balli, E. and Cleyet-Maret, J.C. (2001). Isolation and identification of the most efficient plant growth-promoting bacteria associated to canole *(Brassica napus). Biol. Fertil. Soils.* 33, 152-156. doi: 10.1007/s003740000305.

Bonanomi, G., D'Ascoli, R., Scotti, R., Gaglione, S. A., Caceres, M.G., Sultana, S. *et al.* (2014). Soil quality recovery and crop yield enhancement by combined application of compost and wood to vegetables grown under plastic tunnels. *Agric. Ecosyst. Environ.* 192, 1–7. doi:10.1016/j.agee.2014.03.029.

Bonilla, N., Cazorla, F.M., Martínez-Alonso, M., Hermoso, J.M., González-Fernández, J.J., Gaju, N. *et al.* (2012a). Organic amendments and land management affect bacterial community composition, diversity and biomass in avocado crop soils. *Plant Soil* 357, 215–226. doi:10.1007/s11104-012-1155-1.

Bonilla, N., Gutiérrez-Barranquero, J., Vicente, A. and Cazorla, F. (2012b). Enhancing soil quality and plant health through suppressive organic amendments. *Diversity* 4, 475–491. doi:10.3390/d4040475.

Bonilla, N., Vida, C., Martínez-Alonso, M., Landa, B.B., Gaju, N., Cazorla, F.M., *et al.* (2015). Organic amendments to avocado crops induce suppressiveness and influence the composition and activity of soil microbial communities. *Appl. Environ. Microbiol.* 81, 3405–3418. doi:10.1128/AEM.03787-14.

Brenner, D.J., Krieg, N.R., Staley, J.T., and Garrity, G.M. Eds. (2005). *Bergey's manual of systematic bacteriology*. New York, NY: Springer Science+Business Media, Inc,

Buck, J. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl. Environ. Microbiol.* 44, 4:992-993. doi:10.1111/j.1574-6968.2000.tb09007.x.

Bulluck III, L.R. and Ristaino, J.B. (2001). Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. *Phytopathology* 92, 181-189. doi:10.1094/PHYTO.2002.92.2.181.

Bulluck III, L.R., Brosius, M., Evanylo, G. and Ristaino, J. (2002). Organic and synthetic fertility amendment influence soil microbial physical and chemical properties on organic and conventional farms. *Appl. Soil Ecol.* 19, 147–160. doi:10.1016/S0929-1393(01)00187-1.

Caballero, J.A., Font, R. and Marcilla, A. (1996). Comparative study of the pyrolysis of almond shells and their fractions, holocellulose and lignin. Product yields and kinetics. *Thermochimica Acta* 276, 57-77. doi:10.1016/0040-6031(95)02794-7.

Calderón, C. E., Pérez-García, A., de Vicente, A. and Cazorla, F. M. (2013). The dar genes of *Pseudomonas chlororaphis* PCL1606 are crucial for biocontrol activity via production of the antifungal compound 2-hexyl, 5-propyl resorcinol. *Mol. Plant. Microbe. Interact.* 26, 554–565. doi:10.1094/MPMI-01-13-0012-R.

Calderón, C.E., de Vicente, A. and Cazorla, F. M. (2014). Role of 2-hexyl, 5-propyl resorcinol production by *Pseudomonas chlororaphis* PCL1606 in the multitrophic interactions in the avocado rhizosphere during the biocontrol process. *FEMS Microbiol. Ecol.* 89, 20–31. doi:10.1111/1574-6941.12319.

Calderón, C. E., Ramos, C., de Vicente, A. and Cazorla, F. M. (2015). Comparative genomic analysis of *Pseudomonas chlororaphis* PCL1606 reveals new insight into antifungal compounds involved in biocontrol. *Mol. Plant. Microbe. Interact.* 28, 249–260. doi: 10.1094/MPMI-10-14-0326-FI.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. *et al.* (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi:10.1038/nmeth.f.303.QIIME.

Carvalhais, L.C., Dennis, P.G., Tyson, G.W. and Schenk, P.M. (2012). Application of metatranscriptomics to soil environments. *J. Microbiol. Methods* 91, 246–251. doi:10.1016/j.mimet.2012.08.011.

Castric, P.A. (1975). Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Can. J. Microbiol*. 21, 613-618.

Cazorla, F.M., Duckett, S.B., Bergström, E.T., Noreen, S., Odijk, R., Lugtenberg, B.J.J. *et al.* (2006). Biocontrol of avocado dematophora root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-hexyl 5-propyl resorcinol. *Mol. Plant. Microbe. Interact.* 19, 418–428. doi:10.1094/MPMI-19-0418.

Cazorla, F.M., Romero, D., Pérez-García, A., Lugtenberg, B.J.J., de Vicente, A. and Bloemberg, G.V. (2007). Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *J. Appl. Microbiol.* 103, 1950-1959. doi:10.1111/j.1365-2672.2007.03433.x.

Cernava, T., Aschenbrenner, I. A., Grube, M., Liebminger, S. and Berg, G. (2015). A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria. *Front. Microbiol.* 6, 1–9. doi:10.3389/fmicb.2015.00398.

Chanderbali, A. S., Soltis, D. E., Soltis, P. S., and Wolstenholme, B. N. (2013). "Taxonomy and Botany," in *The Avocado: Botany, Production and Uses*, 2nd Edn, eds B. A. Schaffer, B. N. Wolstenholme, and A. W. Whiley (Oxfordshire: CABI), 31–50. doi: 10.1079/9781845937010.0031

Chin-A-Woeng, T.F.C., Bloemberg, G.V., van der Bij, A.J., van der Drift, K.M. G.M., Schripsema, J., Kroon, B. *et al.* (1998). Biocontrol by phenazine-1-carboxamideproducing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *Mol. Plant. Microbe Interact.* 11, 1069-1077. doi:10.1094/MPMI.1998.11.11.1069 .

Choi, K. H., Kumar, A. and Schweizer, H. P. (2006). A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. *J. Microbiol. Methods* 64, 391–397. doi:10.1016/j.mimet.2005.06.001.

Cong, J., Yang, Y., Liu, X., Lu, H., Liu, X., Zhou, J. *et al.* (2015). Analyses of soil microbial community compositions and functional genes reveal potential consequences of natural forest succession. *Sci. Rep.* 5, 10007. doi:10.1038/srep10007.

Cook, R.J. and Baker, F. Eds. (1983). *The nature and practice of biological control of plant pathogens.* St Paul, MI: The American Phytopathological Society.

Cordero, O.X., Ventouras, L.A., DeLong, E.F. and Polz, M.F. (2012). Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *Proc. Natl. Acad. Sci.* 109, 20059–20064. doi: 10.1073/pnas.1213344109.

Cordovez, V., Carrion, V., Etalo, D., Mumm, R., Zhu, H., van Wezel, G. *et al.* (2015). Diversity and functions of volatile organic compounds produced by *Streptomyces* from a disease-suppressive soil. *Front. Microbiol.* 6: 1081. doi: 10.3389/fmicb.2015.01081.

Cretoiu, M.S., Korthals, G.W., Visser, J.H.M. and van Elsas, J.D. (2013). Chitin amendment increases soil suppressiveness toward plant pathogens and modulates the actinobacterial and oxalobacteraceal communities in an experimental agricultural field. *Appl. Environ. Microbiol.* 79, 5291–5301. doi:10.1128/AEM.01361-13.

Daims, H., Lucker, S. and Wagner, M. (2006). Daime, a novel image analysis program for microbial ecology and biofilm research. *Environ. Microbiol.* 8, 200–213. doi: 10.1111/j.1462-2920.2005.00880.x.

Danovaro, R., Luna,. GM., Dell'Anno, A. and Pietrangeli, B. (2006). Comparison of two fingerprinting techniques, terminal restriction fragment length polymorphism and automated ribosomal intergenic space analysis, for determination of bacterial diversity in aquatic environments. *Appl Environ Microbiol*. 72, 5982–5989. doi: 10.1128/AEM.01361-06.

De Boer, W., Hundscheid, M.P.J., Klein Gunnewiek, P.J.A., de Ridder-Duine, A.S., Thion, C., Van Veen, J.A. *et al.* (2015). Antifungal rhizosphere bacteria can increase as response to the presence of saprotrophic fungi. *PLoS ONE* 10, 9:e0137988. doi:10.1371/journal.pone.0137988.

De Boer, W., Verheggen, P., Klein Gunnewiek, P.J.A., Kowalchuk, G.A. and Van Veen, J.A. (2003). Microbial community composition affects soil fungistasis. *Appl. Environ. Microbiol*. 69, 835–844. doi: 10.1128/AEM.69.2.835-844.2003.

De Gannes, V.D., Endoxic, G. and Hickey, W.J. (2013). Insight into fungal communities in compost revealed by 454-pyrosequencing: implications for human health and safety. *Front. Microbiol*. 4:164. doi:10.3389/fmicb.2013.00164.

Deng, P., Wang, X., Baird, S.M. and Lu, S. (2015). Complete genome of *Pseudomonas chlororaphis* strain UFB2, a soil bacterium with antibacterial activity against bacterial canker pathogen of tomato. *Stand. Genom. Sci.* 10:117. doi: 10.1186/s40793-015-0106-x.

Deng, Y., Yang Y., He, Z. and Zhou, J. (2013). "Molecular ecological network of microbial communities" in *Encyclopedia of Metagenomics*, ed. Nelson, K. (Berlin Heidelberg: Springer-Verlag).

De Roy, K., Marzorati, M., van den Abbeele, P., van de Wiele, T. and Boon, N. (2014). Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities. *Environ. Microbiol.* 16, 1472–1481. doi:10.1111/1462-2920.12343.

De Souza, J.T., Weller, D.M. and Raaijmakers, J.M. (2003). Frequency, diversity and activity of 2, 4-diacetylphloroglucinos-producing fluorescent *Pseudomonas spp*. in Dutch take-all decline soils. *Phytopathology* 93, 1: 54-63. doi: 10.1094/PHYTO.2003.93.1.54.

De Vrieze, M., Pandey, P., Bucheli, T. D., Varadarajan, A. R., Ahrens, C. H., Weisskopf, L. *et al.* (2015). Volatile organic compounds from native potato-associated *Pseudomonas* as potential anti-oomycete agents. *Front. Microbiol.* 6, 1–15. doi:10.3389/fmicb.2015.01295.

De Weert, S., Vermeiren, H., Mulders, I.H.M., Kuiper, I., Hendrickx, N., Bloemberg, G.V. *et al.* (2002). Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. *Mol. Plant Microbe Interact*. 15, 1173-1180. doi: 10.1094/MPMI.2002.15.11.1173.

Dietrich, L. E. P., Price-Whelan, A., Petersen, A., Whiteley, M. and Newman, D. K. (2006). The phenazine pyocyanin is a terminal signalling factor in the *quorum sensing* network of *Pseudomonas aeruginosa*. *Mol. Microbiol.* 61, 1308–1321. doi:10.1111/j.1365-2958.2006.05306.x.

Doran, J.W. and Parkin, T.B. (1994). "Defining and assessing soil quality" in *Defining soil quality for a sustainable environment*, eds. Doran, J.W., Coleman, D.C., Bezdicek, D.F., Stewart, B.A. (Madison, WI: Soil Science Society of America). 35, 3–21.

Doran, J.W. and Parkin, T.B. (1996). "Quantitative indicators of soil quality: a minimum data set" in *Methods for assessing soil quality*, eds. Doran, J.W., and Jones, A.J. (Madison, WI: Soil Science Society of America. 49, 25–37.

Downer, A.J., Menge, J.A. and Pond, E. (2001). Association of cellulolytic enzyme activities in Eucalyptus mulches with biological control of *Phytophthora cinnammomi*. *Phytopathology* 91, 847-855. doi:10.1094/PHYTO.2001.91.9.847.

Drinkwater, L.E., Letourneau, D.K., Workneh, F., van Bruggen, A.H.C. and Shennan, C. (1995). Fundamental differences between conventional and organic tomato agroecosystems in California. *Ecological Applications* 5, 1098-1112. doi:10.2307/2269357.

Edwards, D. and Holt, K. E. (2013). Beginner's guide to comparative bacterial genome analysis using next-generation sequence data. *Microb. Inform. Exp*. 3:2. doi: 10.1186/2042-5783-3-2.

Esfahlan, A.J., Jamei, R. and Esfahlan, R.J. (2010). The importance of almond (*Prunnus amygdalus* L.) and its by-products. *Food Chem.* 120, 3:349-360. doi: 10.1016/j.foodchem.2009.09.063.

Effmert, U., Kalderás, J., Warnke, R. and Piechulla, B. (2012). Volatile mediated interactions between bacteria and fungi in the soil. *J. Chem. Ecol.* 38, 665–703. doi:10.1007/s10886-012-0135-5.

Fernández, R.G., García, C.P., Lavín, A.G. and Bueno de las Heras, J.L. (2012). Study of main combustión characteristics for biomass fuels used in boilers. *Fuel Process. Technol.* 103, 16-26. doi: 10.1016/j.fuproc.2011.12.032.

Fernando, W. G. D., Ramarathnam, R., Krishnamoorthy, A. S. and Savchuk, S. C. (2005). Identification and use of potential bacterial organic antifungal volatiles in biocontrol. *Soil Biol. Biochem.* 37, 955–964. doi:10.1016/j.soilbio.2004.10.021.

Ferrari, B.C., Zhang, C. and van Dorst, J. (2011). Recovering greater fungal diversity from pristine and diesel fuel contaminated sub-Antartic soil through cultivation using both a high and a low nutrient media approach. *Front. Microbiol*. 2:217. doi:10.3389/fmicb.2011.00217.

Freeman, S., Sztejnberg, A. and Chet, I. (1986). Evaluation of *Trichoderma* as a biocontrol agent for *Rosellinia necatrix*. *Plant Soil* 94, 163–170. doi:10.1007/BF02374340.

Frees, D., Brondsted, L. and Ingmer, H. (2013). Bacterial proteases and virulence. *Subcell. Biochem.* 66, 161-192. doi: 10.1007/978-94-007-5940-4_7

Garbeva, P., Hordijk, C., Gerards, S. and de Boer, W. (2014). Volatiles produced by the mycophagous soil bacterium *Collimonas*. *FEMS Microbiol. Ecol.* 87, 639–649. doi:10.1111/1574-6941.12252.

Garbeva, P., Veen, J.A., and van Elsas, J.D. (2004). Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Ann. Rev. Phytopathol*. 42, 243-270. doi:10.1146/annurev.phyto.42.012604.135455.

García-Gutiérrez, L., Romero, D., Zeriouh, H., Cazorla, F.M., Torés, J.A., de Vicente, A., *et al.* (2012). Isolation and selection of plant growth-promoting rhizobacteria as inducers of systemic resistance in melon. *Plant Soil* 358, 201–212. doi:10.1007/s11104-012-1173-z.

Gerhardt, P. Ed. (1994). *Methods for general and molecular bacteriology*. Washington, DC: American Society for Microbiology Press.

Gerlagh, M. (1968). Introduction of *Ophiobolus graminis* into new polders and its decline. *Neth. J. Plant Pathol*. 74, 2:1-97. doi:10.1007/BF02019999.

Ghazali, F.M., Rahman, R.N.Z.A., Salleh, A.B. and Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *Int. Biodeterior. Biodegrad.* 54, 61–67. doi:10.1016/j.ibiod.2004.02.002.

Ghosh, P., Rahinasabapathi, B. and Ma, L.Q. (2015). Phosphorus solubilization and plant growth enhancement by arsenic-resistant bacteria. *Chemosphere* 134, 1-6. doi: 10.1016/j.chemosphere.2015.03.048.

Gkarmiri, K., Finlay, R.D., Alström, S., Thomas, E., Cubeta, M.A. and Högberg, N. (2015). Transcriptomic changes in the plant pathogenic fungus *Rhizoctonia solani* AG-3 in response to the antagonistic bacteria *Serratia proteamaculans* and *Serratia plymuthica*. *BMC Genomics* 16:630. doi 10.1186/s12864-015-1758-z.

Gong, Z.-L., Zhang, C.-F., Jin, R. and Zhang, Y.-Q. (2016). *Steroidobacter flavus* sp. nov., a microcystin-degrading Gammaproteobacterium isolated from soil. *Antonie Van Leeuwenhoek* 109, 1073–1079. doi:10.1007/s10482-016-0706-5.

González-Cortés, J. C., Vega-Fraga, M., Varela-Fregoso, L., Martínez-Trujillo, M., Carreón-Abud, Y., and Gavito, M. E. (2012). Arbuscular mycorrhizal fungal (AMF)communities and land usechange:the conversion of temperate forestto avocado plantations and maize fields in central Mexico. *Fungal Ecol*. 5, 16–23. doi: 10.1016/j.funeco.2011.09.002

González-Sánchez, M.A., de Vicente, A., Pérez-García, A., Pérez-Jiménez, R., Romero, D. and Cazorla, F.M. (2013). Evaluation of the effectiveness of biocontrol bacteria against avocado white root rot occurring under commercial greenhouse plant production conditions. *Biological control* 67, 94-100. doi:10.1016/j.biocontrol.2013.08.009.

Gónzalez-Sánchez, M.A., Pérez-Jiménez, R.M., Pliego, C., Ramos, C., de Vicente, A. and Cazorla, F.M. (2010). Biocontrol bacteria selected by a direct plant protection strategy against avocado white root rot show antagonism as a prevalent trait. *J. Appl. Microbiol*. 109, 65-78. doi:10.1111/j.1365-2672.2009.04628.x.

Grandclément, C., Tanniéres, M., Moréra, S., Dessaus, Y. and Faure, D. (2015). *Quorum quenching*: role in nature and applied developments. *FEMS Microbiol. Rev*. 40, 86-116. doi: 10.1093/femsre/fuv038.

Groβkopf, T. and Soyer, O.S. (2014). Synthetic microbial communities. *Curr. Opin. Microbiol.* 18, 72–77. doi:10.1016/j.mib.2014.02.002.

Gross, H. and Loper, J. E. (2009). Genomics of secondary metabolite production by Pseudomonas spp. *Nat. Prod. Rep*. 26, 1408-1446. doi: 10.1039/B817075B.

Gunapala, N. and Scrow, K.M. (1998). Dynamics of soil microbial biomass and activity in conventional and organic farming systems. *Soil. Biol. Biochem.* 30, 805-816. doi:10.1016/S0038-0717(97)00162-4.

Gupta, U.C., Kening, W.U. and Siyuan, L. (2008). Micronutrients in soils, crops, and livestock. *Earth Sci. Front.* 15:5, 110-125. doi: 10.1016/S1872-5791(09)60003-8.

Gutiérrez-Barranquero, J. A., Pliego, C., Bonilla, N., Calderón, C.E., Pérez-García, A., de Vicente, A. and Cazorla, F.M. (2012). Sclerotization as a long-term preservation methods for *Rosellinia necatrix* strains. *Mycoscience* 53: 460. doi: 10.1007s10267-012-0185-0.

Haas, D. and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3, 307–319. doi:10.1038/nrmicro1129.

Hadar, Y. and Papadopoulou, K.K. (2012). Suppressive composts: microbial ecology links between abiotic environments and healthy plants. *Annu. Rev. Phytopathol.* 50, 133–153. doi:10.1146/annurev-phyto-081211-172914.

Hankin, L. and Anagnostakis, S.L. (1977). Solid media containing carboxymethylcellulose to detect Cx cellulase activity of microorganisms. *J. Gen. Microbiol*. 98, 109–115.

Hammer, P.E., Hill, D.S., Lam, S.T., Van Pée, K.H. and Ligon, J. M. (1997). Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. *Appl. Environ. Microbiol.* 63, 2147–2154.

Haran, S., Schickler, H. and Chet, I. (1996). Molecular mechanism of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. *Microbiol.* 142, 2321-2331.

Hartmann, A., Schmid, M., van Tuinen, D. and Berg, G. (2009). Plant-driven selection of microbes. *Plant Soil* 321, 235-257. doi: 10.1007/s11104-008-9814-y.

Hass, J. H., and Menge, J. A. (1990). VA-mycorrhizal fungi and soil characteristics in avocado (Persea Americana Mill.) orchard soils. *Plant Soil* 127, 207–212. doi: 10.1007/BF00014427

Hawkes, C.V., DeAngelis, K.M. and Firestone, M.K. (2007). "Root interactions with soil microbial communities and processes" in *The Rhizosphere*, eds. Cardon, Z. and Whitbeck, J. (New York, NY: Elsevier). 1, 1-29.

Hazen, T.C., Dubinsky, E.A., DeSantis, T.Z., Andersen, G.L., Piceno, Y.M., Singh, N. *et al.* (2010). Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* 330, 204–208. doi:10.1126/science.1195979.

He, Z., Deng, Y. and Zhou, J. (2012). Development of functional gene microarrays for microbial community analysis. *Curr. Opin. Biotechnol.* 23, 49–55. doi:10.1016/j.copbio.2011.11.001.

Hermoso, J.M., Torres, M.D. and Farré, J.M. (2011). "Effects of organic materials on adult Hass trees" in *Proceedings of the seventh World Avocado Congress*. (Cairne, Australia, 5-9 September).

Hirsch, P.R., Mauchline, T.H., and Clark, I.M. (2013). "Culture-Independent Molecular Approaches to Microbial Ecology in Soil and the Rhizosphere" in *Molecular Microbial Ecology of the Rhizosphere*, ed. De Bruinjn F.J. (Hoboken, NJ: John Wiley & Sons, Inc) 5, 45–55.

Hoffman, M.T. and Arnold, A.E. (2010). Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. *Appl. Environ. Microb*. 76, 4063-4075. doi:10.1128/AEM.02928-09.

Hoitink, H. and Boehm, M. (1999). Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. *Annu. Rev. Phytopathol*. 37, 427-446. doi:10.1146/annurev.phyto.37.1.427.

Hoitink H. and Fahy, P.C. (1986). Basis for the control of soilborne plant pathogens with composts. *Annu. Rev. Phytopathol*. 24, 93–114. doi: 10.1146/annurev.py.24.090186.000521.

Hoitink, H., Stone, A.G. and Han, D.Y. (1991). Suppression of plant diseases by composts. *HortScience* 32, 184-187.

Howe, T.G. and Ward, J.M. (1976). The utilization of Tween 80 as carbon source by *Pseudomonas*. *J. Gen. Microbiol.* 92, 1: 234-235. doi: 10.1099/00221287-92-1-234.

Howell, C.R. and Stipanovic, R.D. (1979). Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* 69, 480-482. doi: 10.1094/Phyto-69-480.

Huber, B., Riedel, K., Kothe, M., Givskov, M., Molin, S. and Eberl, L. (2002). Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. *Mol. Microbiol.* 46:411–426. doi: 10.1046/j.1365-2958.2002.03182.x/.

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C. *et al.* (2016). eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. *Nucleic Acids Res* 44, 286–293. doi:10.1093/nar/gkv1248.

Hunziker, L., Bönisch, D., Groenhagen, U., Bailly, A., Schulz, S. and Weisskopf, L. (2015). *Pseudomonas* strains naturally associated with potato plants produce volatiles with high potential for inhibition of *Phytophthora* infestans. *Appl. Environ. Microbiol.* 81, 821–830. doi:10.1128/AEM.02999-14.

Ishige, T., Tani, A., Sakai, Y. and Kato, N. (2003). Wax ester production by bacteria. *Curr. Opin. Microbiol.* 6, 244-250. doi: 10.1016/S1369-5274(03)00053-5.

Jafari, M., Haghighi, J.A.P. and Zare, H. (2012). Mulching impact on plant growth and production of rainfed fig orchards under drought conditions. *J. Food Agric. Environ.* 10, 428-433.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., van Heuvel, M. and Misset, O. (1994). Bacterial lipases. *FEMS Microbiol. Rev.* 15, 1:29-63. doi: 10.1111/j.1574-6976.1994.tb00121.x.

Janvier, C., Villeneuve, F., Alabouvette, C., Edel-Hermann, V., Mateille, T. and Steinberg, C. (2007). Soil health through soil disease suppression: Which strategy from descriptors to indicators?. *Soil Bio. Biochem.* 39, 1-23. doi:10.1016/j.soilbio.2006.07.001.

Jeewon, R. and Hyde, K.D. (2007). "Detection and diversity of fungi from environmental samples: traditional *versus* molecular approaches" in *Advanced*

techniques in soil microbiology, eds. Varma, A. and Oelmüller, R. (New York, NY: Springer-Verlag Berlin Heidelberg). 1, 1-15.

Jones, O.A.H., Sdepanian, S., Lofts, S., Svendsen, C., Spurgeon, D.J., Maguire, M.L. *et al.* (2014). Metabolomic analysis of soil communities can be used for pollution assessment. *Environ. Toxicol. Chem.* 33, 61–64. doi:10.1002/etc.2418.

Kai, M., Effmert, U., Berg, G. and Piechulla, B. (2007). Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Arch. Microbiol.* 187, 351–360. doi:10.1007/s00203-006-0199-0.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B. and Piechulla, B. (2009). Bacterial volatiles and their action potential. *Appl. Microbiol. Biotechnol.* 81, 1001–1012. doi:10.1007/s00253-008-1760-3.

Kamou, N., Dubey, M., Tzelepis, G., Menexes, G., Papadakis, E., Karlsson, M., Lagopodi, A. and Jensen, D.F. (2016). Investigating the compatibility of the biocontrol agent *Clonostacys rosea* IK726 with prodigiosin-producing *Serratia rubidae* S55 and phenazine-producing *Pseudomonas chlororaphis* ToZa7. *Arch. Microbiol.* 198, 4:369-377. doi: 10.1007/s00203-016-1198-4.

Kato, S., Haruta, S., Cui, Z.J., Ishii, M. and Igarashi, Y. (2005). Stable coexistence of five bacterial strains as a cellulose-degrading community. *Appl. Environ. Microbiol.* 71, 7099–7106. doi:10.1128/AEM.71.11.7099-7106.2005.

Kato, S., Yoshida, R., Yamaguchi, T., Sato, T., Yumoto, I. and Kamagata, Y. (2014). The effects of elevated CO2 concentration on competitive interaction between aceticlastic and syntrophic methanogenesis in a model microbial consortium. *Front. Microbiol.* 5: 575. doi:10.3389/fmicb.2014.00575.

Kim, H.J., Boedicker, J.Q., Choi, J.W. and Ismagilov, R.F. (2008). Defined spatial structure stabilizes a synthetic multispecies bacterial community. *Proc. Natl. Acad. Sci.* USA 105, 18188–18193. doi: 10.1073/pnas.0807935105.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. *et al.* (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* 41:1. doi: 1031093/nar/gks808.

Kloepper, J.W. and Schroth, N. (1980). Plant growth-promoting rhizobacteria and plant growth under gnotobiotic conditions. *Phytopathology* 71, 642-644.

Knowles, C. J. (1976). Microorganisms and cyanide. *Bacteriol. Rev.* 40, 652–680.

Koyama, A., Wallenstein, M., Simpson, R.T. and Moore, J.C. (2014). Soil bacterial community composition altered by increased nutrient availability in Arctic tundra soils. *Front. Microbiol*. 5:516. doi:10.3389/fmicb.2014.00516.

Kyselková, M., Kopecký, J., Frapolli, M., Défago, G., Ságová-Marecková, M., Grundmann, G.L., *et al.* (2009). Comparison of rhizobacterial community composition in soil suppressive or conducive to tobacco black root rot disease. *ISME J.* 3, 1127–1138. doi:10.1038/ismej.2009.61.

Larena, I., Salazar, O., González, V., Julián, M.C. and Rubio, V. (1999). Design of a primer for ribosomal DNA internal transcriber spacer with enhanced specificity for A*scomicetes*. *J. Biotech*. 75, 187-194. doi:10.1016/S0168-1656(99)00154-6.

Larkin, R.P. and Honeycutt, C.W. (2006). Effects of different 3-year cropping systems on soil microbial communities and *Rhizoctonia* diseases of potato. *Phytopathology* 96, 68-79. doi: 10.1094/PHYTO-96-0068.

Latz, E., Eisenhauer, N., Rall, B.C., Scheu, S. and Jousset, A. (2016). Unravelling linkages between plant community composition and the pathogen-suppressive potential of soils. *Sci. Rep.* 6, 23584–23584. doi:10.1038/srep23584.

Lazarovits, G., Tenuta, M. and Conn, K. (2001). Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops. *Australas. Plant. Pathol*. 30, 111-117. doi:10.1071/AP01009.

Lebeis, S.L. (2014). The potential for give and take in plant-microbiome relationships. *Front. Plant Sci.* 5:287. doi:10.3389/fpls.2014.00287.

Liang, Y., He, Z., Wu, L., Deng, Y., Li, G. and Zhou, J. (2010). Development of a common oligonucleotide reference standard for microarray data normalization and comparison across different microbial communities. *Appl. Environ. Microbiol.* 76, 1088–1094. doi:10.1128/AEM.02749-09.

Liu, B., Gumpertz, M.L., Hu, S. and Ristaino, J.B. (2007). Long-term effects of organic and synthetic soil fertility amendments on soil microbial communities and the development of southern blight. *Soil Biol. Biochem.* 39, 2302–2316. doi:10.1016/j.soilbio.2007.04.001.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol*. 63, 4516–4522.

Lockwood, J.L. (1990) "Relation of energy stress to behavior of soil-borne plant pathogens and to disease development" in *Biological control of soil-borne plant pathogens*, eds. Hornby, D. and Cook, R.J. (Wallinford: CAB International). 197-214.

Loong, S.K., Khor, C.S., Jafar, F.L. and AbuBakar, S. (2016). Utility of 16S rDNA sequencing for identification of rare pathogenic bacteria. J. *Clin. Lab. Anal.* 17. doi: 10.1002/jcla.21980

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis II, E. W., Lim, C. K., Shaffer, B. T., Elbourne, L. D. H., Stockwell, V. O., Hartney, S. L., Breakwell, K., Henkels, M. D., Tetu, S. G., Rangel, L. I., Kidarse, T. A., Wilson, N. L., van de Mortel, J. E., Song, C., Blumhagen, R., Radune, D., Hostetler, J. B., Brinkac, L. M., Durkin, A. S., Kluepfel, D. A., Wechter, W. P., Anderson, A. J., Kim, Y. C., Pierson, L. S., III, Lindow, S. E., Kobayashi, D. Y., Raaijmakers, J. M., Weller, D. M., Thomashow, L. S., Allen, A. E. and Paulsen, I. T. (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insight into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genet.* 8, 127: e1002784. doi: 10.1371/journal.pgen.1002784.

López, R., Burgos, P., Hermoso, J.M., Hormaza, J.I., and González-Fernández, J.J. (2014). Long term changes in soil properties and enzyme activities after almond shell mulching in avocado organic production. *Soil Tillage Res.* 143, 155–163. doi:10.1016/j.still.2014.06.004.

López-Herrera, C.J., Pérez-Jiménez, R.M., Zea-Bonilla, T., Basallote-Ureba, M.J. and Melero-Vara, J.M. (1998). Soil solarization in established avocado trees for control of *Dematophora necatrix*. *Plant Dis.* 82, 1088–1092. doi:10.1094/PDIS.1998.82.10.1088.

López-Herrera, C.J. and Zea-Bonilla, T. (2007). Effects of benomyl, carbendazim, fluazinam and thiophanate methyl on white root rot of avocado. *Crop Prot.* 26, 1186–1192. doi:10.1016/j.cropro.2006.10.015.

Lugtenberg, B. and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.,* 63, 541-556. doi: 10.1146/annurew.micro.62.081307.162918.

Lynch, J.M. and Whipps, J.M. (1990). Substrate flow in the rhizosphere. *Plant and Soil* 129, 1-10. doi:10.1007/BF00011685.

Magno-Pérez-Bryan, M.C., Martínez-García, P.M., Hierrezuelo, J., Rodríguez-Palenzuela, P., Arrebola, E., Ramos, C. *et al.* (2015). Comparative genomics within the *Bacillus* genus reveal the singularities of two robust *Bacillus amyloliquefaciens* biocontrol strains. *Mol. Plant. Microbe Interact*. 10, 1102-1116. doi: 10.1094/MPMI-02-15-0023-R.

Malajczuk, N. (1979) "Biological suppression of *Phytophthora cinnamomi* in eucalypts and avocados in Australia" in *Soil-borne Plant Pathogens*, eds. Schippers, B. and Gams, W. (London: Academic Press).

Malajczuk, N. (1983). "Microbial antagonism to Phytophthora" in *Phytophthora, its biology, taxonomy, ecology and pathology*, eds. Erwin, D.C., Bartnicki-Garcia, S. and Tsao, P.H. (St. Paul, MN: American Phytopathological Society).

Mandelbaum, R. and Hadar, Y. (1990). Effects of available carbon source on microbial activity and suppression of *Pythium aphanidermatum* in compost and peat container media. *Phytopathology* 80, 794–804. doi: 10.1094/Phyto-80-794.

Matas, I.M., Castañeda-Ojeda, M.P., Aragón, I.M., Antúnez-Lamas, M., Murillo, J., Rodríguez-Palenzuela, P. *et al.* (2014). Translocation and functional analysis of *Pseudomonas savastanoi* pv. *savastanoi* NCPPB 3335 type III secretion system effectors reveals two novel effector families of the *Pseudomonas syringae* complex. *Mol. Plant. Microbe Interact.* 27:5, 424-436. doi: 10.1094/MPMI-07-13-0206-R.

Masry, M.H., Khalil, A.I., Hassouna, M.S. and Ibrahim, H.A.H. (2002). *In situ* and *in vitro* suppressive effect of agricultural composts and their water extracts on some phytopathogenic fungi. *World J. Microbiol. Biotech*. 18, 551–558. doi: 10.1023/A:1016302729218.

McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M. *et al.* (1997). *Quorum sensing* and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiol*. 143: 3703-3711. doi: 10.1099/00221287-143-12-3703.

Mendes, R., Garbeva, P. and Raaijmakers, J.M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiol. Rev.* 37, 634–663. doi:10.1111/1574-6976.12028.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Shneider, J.H.M., *et al.* (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097-1100. doi:10.1126/science.1203980.

Miao, C.P., Mi, Q.L., Qiao, X.G., Zheng, Y.K., Chen, Y.W., Xu, L. *et al.* (2015). Rhizospheric fungi of *Panax notoginseng*: diversity and antagonism to host phytopathogens. *J. Ginseng Res*. 40, 2:127-134. doi:10.1016/j.jgr.2015.06.004.

Mitri, S. and Foster, K. R. (2013). The genotypic view of social interactions in microbial communities. *Annu. Rev. Genet.* 47, 247–273. doi:10.1146/annurev-genet-111212-133307.

Moore-Gordon, C., Cowan, A.K. and Wolstenholme, B.N. (1997). "Mulching of avocado orchards to increase Hass yield and fruit size and boost financial rewards a three season summary of research findings" in *South African Avocado Growers' Association Yearbook*. (Tzanee, SAAGA) 20, 46-49.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H. and Wawer, C. (2004). "Denaturing gradient gel electrophoresis (DGGE) in microbial ecology" in *Molecular Microbial Ecology,* eds. Kowalchuk, G.A., Bruinjn, F.J., Heas, I.M., Akkermans, A.D.L., van Elsas, J.D. (Dordrecht: Kluwer Academic Publications) 1, 743–770.

Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700. doi:0099-2240/93/030695-06$02.00/0.

Murthy, N. and Bleakley, B. (2012). Simplified method of preparing colloidal chitin used for screening of chitinase-producing microorganisms. *The Internet Journal of Microbiology* Vol. 10, 2.

Neher, D.A., Weicht, T.R., Bates, S.T., Left, J.W. and Fierer, N. (2013). Changes in bacterial and fungal communities across compost recipes, preparation method, and composting times. *PLoS ONE* 8, 11: e79512. doi:10.1371/journal.pone.0079512.

Nitta, T. (1991). Diversity of root fungal floras: its implications for soil-borne diseases and crop growth. *Jpn. Agric. Res*. 25, 6-11.

Ortíz-Bermúdez, P., Hirth, K.C., Srebotnik, E. and Hammel, K.E. (2007) Chlorination of lignin by ubiquitous fungi has a likely role in global organichloride production. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3895-3900. doi:10.1073/pnas.0610074104.

Pane, C., Piccolo, A., Spaccini, R., Celano, G., Villecco, D. and Zaccardelli, M. (2013). Agricultural waste-based composts exhibiting suppressivity to diseases caused by the phytopathogenic soil-borne fungi *Rhizoctonia solani* and *Sclerotinia minor*. *Appl. Soil Ecol.* 65, 43–51. doi:10.1016/j.apsoil.2013.01.002.

Parsed, M. R., and Greenberg, E. P. (2000). Acyl-homoserine lactone *quorum sensing* in gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8789–8793. doi:10.1073/pnas.97.16.8789.

Paul, E.A. and Clark, F.E. Eds (1996). *Soil microbiology and biochemistry*, 2nd Ed. San Diego, California: Academic Press, Inc.

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Green, S. and Dangl, J. L. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi:10.1073/pnas.1302837110.

Pehlivan, E., Altun, T., Cetin, S. and Bhanger, M. I. (2009) Lead sorption by waste biomass of hazelnut and almond shell. *J. Hazard. Mater*. 167, 1203-2128. doi:10.1016/j.jhazmat.2009.01.126.

Pérez-García, A., Romero, D. and de Vicente, A. (2011). Plant protection and growth stimulation by microorganisms: biotechnological applications of *Bacilli* in agricultura. *Curr. Opin. Biotechnol.* 22, 2: 187-193. doi: 10.1016/j.copbio.2010.12.003.

Pérez-Jiménez, R.M. (2008). Significant avocado diseases caused by fungi and oomycetes. *Eur. J. Plant Sci. Biotechnol.* 2, 1–24.

Pierson, L. S. and Pierson, E. A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and biotechnological processes. *Appl. Microbiol. Biotechnol.* 86, 1659–1670. doi:10.1007/s00253-010-2509-3.

Pliego, C., Cazorla, F.M., González-Sánchez, M.A, Pérez-Jiménez, R.M., de Vicente, A. and Ramos, C. (2007). Selection for biocontrol bacteria antagonistic toward *Rosellinia necatrix* by enrichment of competitive avocado root tip colonizers. *Res. Microbiol.* 158, 463–470. doi:10.1016/j.resmic.2007.02.011.

Pliego, C., De Weert, S., Lamers, G., de Vicente, A., Bloemberg, G.V, Cazorla, F.M. *et al.* (2008). Two similar enhanced root-colonizing *Pseudomonas* strains differ

largely in their colonization strategies of avocado roots and *Rosellinia necatrix* hyphae. *Environ. Microbiol.* 10, 3295–3304. doi:10.1111/j.1462-2920.2008.01721.x.

Pliego, C., Kanematsu, S., Ruano-Rosa, D., de Vicente, A, López-Herrera, C., Cazorla, F.M. *et al.* (2009). GFP sheds light on the infection process of avocado roots by *Rosellinia necatrix. Fungal Genet. Biol.* 46, 137–145. doi:10.1016/j.fgb.2008.11.009.

Pliego, C., López-herrera, C., Ramos, C. and Cazorla, F. M. (2012). Developing tools to unravel the biological secrets of *Rosellinia necatrix*, an emergent threat to woody crops. *Mol. Plant Pathol.* 13, 226–239. doi:10.1111/J.1364-3703.2011.00753.X.

Popova, A.A., Koksharova, O.A., Lipasova, V.A., Zaitseva, J.V., Katkova-Zhukotskaya, O.A., Eremina, S.I. *et al.* (2014). Inhibitory and toxic effects of volatiles emitted by strains of *Pseudomonas* and *Serratia* on growth and survival of selected microorganisms, *Caenorhabditis elegans*, and *Drosophila melanogaster*. *Biomed. Res. Int*. 125704. doi:10.1155/2014/125704.

Postma, J., Sheper, R.W.A. and Schilder, M.T. (2010). Effect of successive cauliflower plantings and *Rhizoctonia solani* AG 2-1 inoculations on disease suppressiveness of a suppressive and conducive soil. *Soil Biology and Biochemistry* 42, 804-812. doi:10.1016/j.soilbio.2010.01.017.

Postma, J., Willemsen-de Klein, M.J. and van Elsas, J.D. (2000). Effect of the indigenous microflora on the development of root and crown rot caused by *Pythium aphanidermatum* in cucumber grown on rockwool. *Phytopathology* 90, 125-133. doi:10.1094/PHYTO.2000.90.2.125.

Raaijmakers, J.M. and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Ann. Rev. Phytopathol*. 50, 20:1-22. doi:10.1146/annurev-phyto-081211-172908.

Raaijmakers, J. M., de Bruijn, I., Nybroe, O., and Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev.* 34, 1037–1062. doi:10.1111/j.1574-6976.2010.00221.x.

Raaijmakers, J.M., Vlami, M. and de Souza, J.T. (2002). Antibiotic production by bacterial biocontrol agents. *Antonie Van Leeuwenhoek*, 81, 537-547. doi: 10.1023/A:1020501420831.

Robledo, J.D. and Hermoso, J.M. Eds. (2009). *Frutos tropicales en la costa andaluza*. Las Rozas, Madrid: Ediciones Norma-Capitel.

Rovira, A.D. and Wildermuth, G.B. (1981). "The nature and mechanisms of suppression" in *Biology and control of take all*, eds. Asher, M.J.C. and Shipton, P. (London: Academic) 385-415.

Ruano-Rosa, D., and López-Herrera, C. J. (2009). Evaluation of Trichoderma spp. As biocontrol agents against avocado white root rot. *Biol. Cont.* 51, 66–71. doi: 10.1016/j.biocontrol.2009.05.005

Ruano-Rosa, D., Cazorla, F.M., Bonilla, N., Martín-Pérez, R., de Vicente, A. and López-Herrera, C.J. (2014). Biological control of avocado white root rot with combined applications of *Trichoderma* spp. and rhizobacteria. *Eur. J. Plant Pathol.* 138, 751–762. doi:10.1007/s10658-013-0347-8.

Ryu, C.M., Hu, C.H., Locy, R.D. and Kloepper, J.W. (2005). Study of mechanisms for plant growth promotion elicited by rhizobacteria in *Arabidopsis thaliana*. *Plant Soil* 268, 285-292. doi: 10.1007/x11104-004-0301-9

Sands, D.C. and Rovira, D. (1970). Isolation of fluorescent pseudomonads with a selective medium. *Appl. Microbiol*. 20, 3:513-514.

Schrimer, A., Rude, M.A., Li, X., Popova, E., and del Cardayre, S.B. (2010). Microbial biosynthesis of alkanes. *Science* 329, 559–562. doi:10.1126/science.1187936.

Selin, C., Dilantha Fernando, W.G. and de Kievit, T. (2011). The PhzI/PhzR *quorum-sensing* system is required for pyrrolnitrin and phenazine production, and exhibits cross-regulation with RpoS in *Pseudomonas chlororaphis* PA23. *Microbiol.* 158, 896-907. doi: 10.1099/mic.0.054254.

Shen, X.Y., Cheng, Y.L., Cai, C.J., Fan, L., Gao, J. *et al.* (2014) Diversity and antimicrobial activity of culturable endophytic fungi isolated from Moso Bamboo seeds. *PLoS ONE* 9, 4: e95838. doi:10.1371/journal.pone.0095838.

Shrestha, K., Shrestha, P., Walsh, K.B, Harrower, K.M. and Midmore, D.J. (2011) Microbial enhancement of compost extracts based on cattle rumen content. compost-characterisation of a system. *Bioresource Technology* 102,8027–8034. doi:10.1016/j.biortech.2011.06.076.

Silby, M. W., Winstanley, C., Godfrey, S. A. C., Levy, S. B. and Jackson, R. W. (2011). *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiol. Rev.* 35, 652–680. doi:10.1111/j.1574-6976.2011.00269.x.

Sokal, R.R. and Rohl, F.J. (1986). *Introducción a la bioestadística*, S.A. Barcelona: Reverté.

Song, H., Ding, M.Z., Jia, X.Q., Ma, Q. and Yuan, Y.J. (2014). Synthetic microbial consortia: from systematic analysis to construction and applications. *Chem. Soc. Rev.* 43, 6954–6981. doi:10.1039/c4cs00114a.

Stein, T. (2005). *Bacillus subtilis* antibiotics: Structures, syntheses and specific functions. *Mol. Microbiol.* 56, 845–857. doi:10.1111/j.1365-2958.2005.04587.x.

Stone, A.G., Scheuerell, S.J. and Darby, H.M. (2004). "Suppression of soilborne diseases in field agricultural systems: organic matter management, cover cropping, and other cultural practices" in *Soil organic matter in sustainable agriculture*, eds. Magdoff, F. and Well, R.R. (Boca Ratón, FL: CRC Press). 131-177.

Stoylar, S., van Dien, S., Hillesland, K.L., Pinel, N., Lie, T.J., Leigh, J.A, *et al.* (2007). Metabolic modeling of a mutualistic microbial community. *Mol. Syst. Biol.* 3:92. doi:10.1038/msb4100131.

Stutz, E.W., Défago, G. and Kern, H. (1986). Naturally occurring fluorescent pseudomonads involved in suppression of black root rot of tobacco. *Phytopathology* 76, 181–185. doi: 10.1094/Phyto-76-181.

Sylvester-Bradley, R., Asakawa, N., la Torraca, S., Magalhaes, F.M.M, Oliveira, L. and Pereira, R.M. (1982). Levantamento quantitativo de microrganismos solubilizadores de fosfatos na rizosfera de gramineas e leguminosas forrageiras na Amazônia. *Acta Amaz*. 12, 15-22.

Teague, B.B.P. and Weiss, R. (2015). Synthetic communities, the sum of parts. *Science* 349, 924–925. doi:10.1126/science.aad0876.

Termorshuizen, A.J., van Rijn, E., van der Gaag, D.J., Alabouvette, C., Chen, Y., Lagerföf, J. *et al.* (2006). Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. *Soil Biol. Biochem.* 38, 2461-2477. doi:10.1016/j.soilbio.2006.03.002.

Thomas, T., Gilbert, J. and Meyer, F. (2012). Metagenomics, a guide from sampling to data analysis. *Microb. Inform. Exp.* 2:3. doi:10.1186/2042-5783-2-3.

Tienda, S., Vida, C., Arrebola, E., de Vicente, A. and Cazorla, F.M. (2016). The rhizobacteria *Pseudomonas chlororaphis* PCL1606 does not have PGPR activity as additional mechanisms to biocontrol. *IOBC-WPRS Bulletin* 117, p. 21.

Torsvik, V. and Ovreas, L. (2002) Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5, 3:240–324. doi:10.1016/S1369-5274(02)00324-7.

Tu, Q., Yu, H., He, Z., Deng, Y., Wu, L., Van Nostrand, J.D., *et al.* (2014). GeoChip 4: a functional gene-array-based high-throughput environmental technology for microbial community analysis. *Mol. Ecol. Resour.* 5, 1–15. doi:10.1111/1755-0998.12239.

Vacheron, J., Desbrosses, G., Bouffaud, M.L., Touraine, B., Moënne-Loccoz, Y., Muller, D. *et al.* (2013). Plant growth-promoting rhizobacteria and root system functioning. *Front. Plant Sci*. 4:356. doi: 10.3389/fpls.2013.00356.

Valverde, M., Madrid, R., García, A. L., del Amor, F. M., and Rincón, L. (2013). Use of almond shell and almond hull as substrate for sweet pepper cultivation. Effect on fruit yield and mineral content. *Span. J. Agric. Res.* 11, 164–172. doi: 10.5424/sjar/2013111-3566

Van Elsas, J.D. and Postma, J. (2007). "Suppression of soil-borne phytopathogens by compost" in *Compost Science and Technology*, eds. Diaz, L.F., de Bertoldi, M., Bidlingmaier, W., Stentiford, E. (Amsterdam, Elsevier). 8, 201-214.

Van Elsas, J.D., Costa, R., Jansson, J., Sjöling, S., Bailey, M., Nalin, R. *et al.* (2008). The metagenome of disease suppressive soils, experiences from the METACONTROL project. *Trends in Biotecnology* 26, 591-601. doi:10.1016/j.tibtech.2008.07.004.

Van Elsas, J. D., Jansson, J. K. and Trevors, T. Eds. (2007). *Modern Soil Microbiology II*. Boca Raton, Florida: CRC Press, Taylor & Francis Group, LLC.

Van Os and van Ginkel. (2001). Suppression of *Pythium* root rot in bulbous iris in relation to biomass and activity of the soil microflora. *Soil Biol. Biochem*. 32, 1447-1454. doi:10.1016/S0038-0717(01)00053-0.

Van Veen, J. A., van Oberbeek, L. S., and van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. Rev*. 61, 121–135.

Vergunst, A., Meijer, A.H., Renshaw, S.A. and O'Callaghan, D. (2010). *Burkholderia cenocepacia* creates an intramacrophage replication niche in zebrafish embryos, followed by bacterial dissemination and establishment of systemic infection. *Infect. Immun.* 78, 1495-1508. doi: 10.1128/IAI.00743-09.

Viebahn, M.C., Veeman, C., Wernars, K., van Loon, L.C., Smith, E. and Bakker, P.A.H.M. (2005). Assessment of differences in *Ascomycete* communities in the rhizosphere of field-grown wheat and potato. *FEMS Microbiology Ecology* 53, 245-253. doi:10.1016/j.femsec.2004.12.014.

Vida, C., Bonilla, N., de Vicente, A. and Cazorla, F.M. (2016). Microbial profiling of a suppressiveness-induced agricultural soil amended with composted almond shells. *Front. Microbiol.* 7:4. doi:10.3389/fmicb.201600004.

Vida, C., Cazorla, F.M. and de Vicente, A. (2017a) Characterization of biocontrol strains after an amendment with composted almond shells from a suppressiveness-induced soil. *Res. Microbiol.* Under review.

Vida, C., de Vicente, A. and Cazorla, F.M. (2017b). Draft genome of the rhizobacterium *Pseudomonas chlororaphis* PCL1601, displaying biocontrol against soilborne phytopathogens. *Genome Announc.* In press.

Wagner, L., Stielow, B., Hoffmann, K., Petkovits, T., Papp, T., Vágvölgyi, C. *et al.* (2013). A comprehensive molecular phylogeny of the *Mortierellales* (*Mortierellomycotina*) based on nuclear ribosomal DNA. *Persoonia* 30, 77-93. doi:10.3767/003158513X666268.

Walsh, G.A., Murphy, R.A., Killeen, G.F., Headon, D.R. and Power, R.F. (1995). Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. *J. Anim. Sci*. 73, 1074-1076.

Wang, H.B., Zhang, Z.X., Li, H., He, H.B., Fang, C.X., Zhang, A.J. *et al.* (2011). Characterization of metaproteomics in crop rhizospheric soil. *J. Proteome Res.* 10, 932–940. doi:10.1021/pr100981r.

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R. *et al.* (2015). antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* 43, 237–243. doi:10.1093/nar/gkv437.

Weller, D.M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology* 97, 250–256. doi:10.1094/PHYTO-97-2-0250.

Weller, D.M., Raaijmakers, J.M., Gardener, B.B. M. and Thomashow, L.S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348. doi:10.1146/annurev.phyto.40.030402.110010.

Wolstenholme, B.N., Moore-Gordon, C.S., and Cowan, A.K. (1997). "Orchard mulching effects on avocado fruiting" in *Proceedings from the Australian Avocaco Growers' Association Conference'97: Searching for Quality*, ed. Cutting, I.G. (Rotorna, New Zealand, 23-26 September). 119-130.

Workneh, F. and van Bruggen, A.H.C. (1994). Microbial densitiy, composition, and diversity in organically and conventionally managed rhizosphere soil in relation to suppression of corky root of tomatoes. *Appl. Soil Ecol*. 1, 219-230. doi:10.1016/0929-1393(94)90013-2.

Wu, X., Monchy, S., Taghavi, S., Zhu, W., Ramos, J. and van der Lelie, D. (2011). Comparative genomics and functional analysis of niche-specific adaptation in *Pseudomonas putida*. *FEMS Microbiol. Rev.* 35, 299–323. doi:10.1111/j.1574-6976.2010.00249.x.

Xie, J., He, Z., Liu, X., Liu, X., Van Nostrand, J. D., Deng, Y. *et al.* (2011). GeoChip-based analysis of the functional gene diversity and metabolic potential of microbial communities in acid mine drainage. *Appl. Environ. Microbiol.* 77, 991–999. doi:10.1128/AEM.01798-10.

Xue, C., Penton, R., Shen, Z., Zhang, R., Huang, Q., Li, R. *et al.* (2015). Manipulating the banana rhizosphere microbiome for biological control of Panama disease. *Sci. Rep.* 5, 11124; doi: 10.1038/srep11124.

Xue, K., Wu, L., Deng, Y., He, Z., Van Nostrand, J., Robertson, P.G. *et al.* (2013). Functional gene differences in soil microbial communities from conventional, low-input, and organic farmlands. *Appl. Environ. Microbiol.* 79, 1284–1292. doi:10.1128/AEM.03393-12.

Zaccardelli, M., de Nicola, F., Villecco, D. and Scotti, R. (2013). The development and suppressive activity of soil microbial communities under compost amendment. *Journal of Soil Science and Plant Nutrition* 13, 730-742.

Zarraonaindia, I., Owens, S.M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S. *et al.* (2015). The soil microbiome influences grapevine-associated microbiota. *mBio* 6: e02527-14. doi: 10.1128/mBio.02527-14.

Zgur-Bertok, D. (2013). DNA damage repair and bacterial pathogens. *PLoS pathog.* 9, 11: e1003711. doi:10.1371/journal.ppat.1003711.

Zhang, Y., Fernando, W.G.D., de Kievit, T.R., Berry, C., Daayf, F. and Paulitz, T.C. (2006). Detection of antibiotic-related genes from bacterial biocontrol agents with polymerase chain reaction. *Can. J. Microbiol*. 52, 476-481. doi:10.1139/w05-152.

Zhang, Y., Lu, Z., Liu, S., Yang, Y., He, Z., Ren, Z. *et al.* (2013). GeoChip-based analysis of microbial communities in alpine meadow soils in the Qinghai-Tibetan plateau. *BMC Microbiol.* 13:72. doi:10.1186/1471-2180-13-72.

Zhou, C.S., Mo, M.H., Gu, Y.Q., Zhou, J.P. and Zhang, K. Q. (2007). Possible contributions of volatile-producing bacteria to soil fungistasis. *Soil Biol. Biochem.* 39, 2371–2379. doi:10.1016/j.soilbio.2007.04.009.

Web sites:

http://antismash.secondarymetabolites.org/ (AntiSMASH)

http://bioinfogp.cnb.csic.es/tools/venny/ (Venny tool)

http://cg.ezbiocloud.net/ (EZbiocloud)

http://ieg.ou.edu/microarray/ (GeoChip® pipeline)

http://www.asajamalaga.com/?n=1596 (ASAJA)

https://www.avocadocentral.com (Avocado Central)

http://www.chemspider.com/ (ChemSpider database)

http://www.fao.org/faostat/en/#data (FAOSTAT)

http://www.glomics.com/gch-tech.html (GeoChip®)

ANNEX

AEM
Journals.ASM.org

Organic Amendments to Avocado Crops Induce Suppressiveness and Influence the Composition and Activity of Soil Microbial Communities

Nuria Bonilla,[a] Carmen Vida,[a] Maira Martínez-Alonso,[b] Blanca B. Landa,[c] Nuria Gaju,[b] Francisco M. Cazorla,[a] Antonio de Vicente[a]

Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora," Universidad de Málaga, Consejo Superior de Investigaciones Científicas, and Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain[a]; Departamento de Genética y Microbiología, Facultad de Biociencias, Universidad Autónoma de Barcelona, Bellaterra, Spain[b]; Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, Consejo Superior de Investigaciones Científicas, Finca Alameda del Obispo, Córdoba, Spain[c]

One of the main avocado diseases in southern Spain is white root rot caused by the fungus *Rosellinia necatrix* Prill. The use of organic soil amendments to enhance the suppressiveness of natural soil is an inviting approach that has successfully controlled other soilborne pathogens. This study tested the suppressive capacity of different organic amendments against *R. necatrix* and analyzed their effects on soil microbial communities and enzymatic activities. Two-year-old avocado trees were grown in soil treated with composted organic amendments and then used for inoculation assays. All of the organic treatments reduced disease development in comparison to unamended control soil, especially yard waste (YW) and almond shells (AS). The YW had a strong effect on microbial communities in bulk soil and produced larger population levels and diversity, higher hydrolytic activity and strong changes in the bacterial community composition of bulk soil, suggesting a mechanism of general suppression. Amendment with AS induced more subtle changes in bacterial community composition and specific enzymatic activities, with the strongest effects observed in the rhizosphere. Even if the effect was not strong, the changes caused by AS in bulk soil microbiota were related to the direct inhibition of *R. necatrix* by this amendment, most likely being connected to specific populations able to recolonize conducive soil after pasteurization. All of the organic amendments assayed in this study were able to suppress white root rot, although their suppressiveness appears to be mediated differentially.

Soil organic matter is fundamental to the long-term sustainability of agroecosystems, and it plays a critical role in global biochemical cycles (1). In the agriculture of the past century, the use of manure and other organic waste material was progressively supplanted by synthetic agrochemicals. This change has led to a decline in soil structure and health that is often related to an increase in plant diseases (2). Therefore, the use of organic amendments has reemerged as an environmentally benign alternative to improve soil quality. This practice has been related to increases in crop yield and plant health and the enhancement of natural suppressiveness of soil against several phytopathogens (3). However, the type and nature of the amendment and the rate of application must be carefully selected for each specific pathosystem. Thus, several authors have reported on the possible negative effects of organic amendments in certain conditions, such as phytotoxicity and increased disease incidence (4–7).

The use of organic amendments or mulches in avocado crops (*Persea americana* Mill.) has produced beneficial effects such as increased root growth and health, reduced plant stress in adverse climatic conditions, and increased avocado yield (8–10). Several organic amendments have shown an obvious suppressive effect against the oomycete *Phytophthora cinnamomi*, the pathogen that causes the widespread *Phytophthora* root rot of avocado (11, 12). However, there is a lack of information about the potential positive or adverse effects of organic amendments on white root rot in avocado caused by *Rosellinia necatrix*. This fungus mostly affects avocado crops in Mediterranean countries, where the presence of the pathogen in the soil, together with the favorable environmental conditions, has turned this disease into one of the main limiting factors for avocado production (13).

The quantity and quality of organic matter input affect both the physicochemical properties of the soil and biotic factors related to soil microbiota such as microbial biomass and diversity, community structure, and soil activities (14–20). The suppressive effects of organic amendments and compost appear related to their influence on soil microbiota because soil pasteurization usually leads to the loss of suppressiveness (21, 22). However, the specific nature of disease suppression is unknown in most cases, and the particular mechanisms involved have not yet been identified (23). To determine which amendments have potential suppressive capability, it is important to identify the microbial populations and associated processes that could account for disease suppression (24).

Several attempts have been made to identify the key factors involved in soil suppressiveness and to find predictive parameters

Received 18 November 2014 **Accepted** 1 March 2015

Accepted manuscript posted online 13 March 2015

Citation Bonilla N, Vida C, Martínez-Alonso M, Landa BB, Gaju N, Cazorla FM, de Vicente A. 2015. Organic amendments to avocado crops induce suppressiveness and influence the composition and activity of soil microbial communities. Appl Environ Microbiol 81:3405–3418. doi:10.1128/AEM.03787-14.

Editor: D. Cullen

Address correspondence to Antonio de Vicente, adevicente@uma.es.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.03787-14.

doi:10.1128/AEM.03787-14

TABLE 1 Main characteristics of the treatments used in the plant assays

Treatment	Code	Composition and composting procedure	Composting time	
			Assay 1	Assay 2
Unamended control	UC	No organic amendment was added		
Almond shells	AS	Commercial almond shells derived from almond industry were piled and traditionally composted; the compost pile was only watered with rainwater	5 yr	1 yr
Pruning waste	PW	Avocado wood derived from pruning waste was finely chopped and composted; the compost pile was watered and turned for aeration every month	5 mo	5 mo
Yard waste	YW	Yard and garden wastes (mostly grass) collected from gardens of the area and composted; the compost pile was watered and turned for aeration every month	5 mo	5 mo

for the suppressive potential of soil organic amendments (25–27). Many authors have correlated the suppression of certain plant diseases with organic parameters related to physicochemical properties (28, 29), microbial biomass (30, 31), microbial diversity (23, 32), microbial community composition (33, 34), microbial overall activity (30, 35), metabolic and enzymatic profiles (34, 36), and particular enzymatic activities (37, 38). Even if certain of these parameters are better predictors than others, none is universally related to disease suppression, and their correlation with suppressiveness is largely dependent on the pathosystem, the type of amendment used, and the environmental conditions (26, 27). In this sense, each type of organic amendment must still be empirically tested for different pathogens, crops, and environments (39).

The availability of suitable methodologies for obtaining detailed information about soil community composition and functioning in each of the suppressive models is a key to unraveling the variety of subjacent mechanisms of disease suppression (40). Limitations and biases of culture-based methods for studying soil microorganisms have been overcome by molecular methods that can analyze a large portion of the nonculturable microorganisms in soil (41). Denaturing gradient gel electrophoresis (DGGE) is a well-established fingerprint method in microbial ecology (42), and it has been successfully used in comparative analyses to study the influence of a wide range of parameters and conditions on the microbial community composition of soil and the rhizosphere (14, 43–47). However, microbial activities and functional diversity might be as important as phylogenetic traits when studying soil microbial communities (48, 49). Organic amendments are known to affect soil functional diversity (18), and the analysis of changes in metabolic and enzymatic abilities can potentially discriminate between suppressive and conductive soils (34, 36).

The objective of the present study was to assess the effects of three different vegetal organic amendments on the development of avocado white root rot caused by *R. necatrix* and to monitor their influence on plant growth under controlled conditions. Microbial communities in the soil and rhizosphere of amended plants were characterized and compared to those of control plants using a polyphasic approach. This analysis includes both culture-based and culture-independent methods for assessing chemical and biological parameters that are potentially involved in the effects of organic amendments on the development of avocado white root rot.

MATERIALS AND METHODS

Greenhouse inoculation assay. An experimental microplot platform was designed and constructed for the plant assays to mimic semifield conditions. The greenhouse was built as an open structure with double roofing to allow air passage for improved ventilation, and the microplots (35 liter plant pots) were earthed up in a white gravel bank to reduce oscillation of the soil temperature (see Fig. S1 in the supplemental material). Two independent 1-year-long experiments were conducted and are named in the present study as assay 1 and assay 2. Two-year-old commercial avocado seedling plants (cv. Topa-Topa) were transplanted to 35 liter pots filled with a blend (1:1) of disinfested natural soil and peat. All plants, except for those in the control treatment, had the top layer of soil mulched with 19 liters of one of the experimental organic amendments (or disinfected soil for the control) as described in Fig. S1B in the supplemental material. Seventeen avocado plants were used for each of the four treatments assayed in these studies as listed in Table 1. After application, the organic amendments matured for six additional months in the greenhouse as the plants grew, and then the soil was inoculated using wheat grains colonized by *R. necatrix* as described by Sztejnberg and Madar (50). Four holes per pot were made on the soil surface using a punch, and 16 g of wheat colonized with *R. necatrix* strain CH53 was distributed in the holes before filling with the surrounding soil.

Eleven plants for each treatment were inoculated with the pathogen, and the remaining six noninoculated plants were used as a control. Plant disease was monitored for 6 months after inoculation. At this point, soil and rhizosphere samples were taken from the control plants of each of the assayed treatments to study the effect of the organic amendments on the microbial community. No samples were taken from the inoculated plants because most of them were dead several weeks before the end of the assay.

Plant growth. Plant growth was monitored in both the inoculated and noninoculated plants. The circumference of the trunk was measured at a height of 15 cm above the ground and used to calculate the trunk cross-sectional area. The lateral branches were removed to maintain apical dominance and the lengths of the main and lateral stems were measured and summed for calculation of the total growth of lateral branches. Variation in plant size after 9 months of the experiment was used to calculate three plant growth parameters: increase in plant height (%), increase in trunk cross-sectional area (%), and total growth of lateral branches (cm).

Disease assessment. Disease progression was measured by evaluating the aerial symptoms of white root rot in symptomatic plants using a symptom scale modified from Ruano-Rosa et al. (51): 0, healthy plant; 1, plant with first symptoms of wilt; 2, overall wilted plant; 3, wilted plant with first symptoms of leaf desiccation; and 4, completely dried plant (dead plant) (see Fig. S2 in the supplemental material). The disease index (DI) in each treatment was calculated according to the method described by Cazorla et al. (52). The experiment was considered finished 165 days postinoculation in both of the assays. For statistical comparison of the treatments, the area under the disease progress curve (AUDPC) was calculated for each plant (51, 52).

Chemical analysis of the soil. Three bulk soil samples from each treatment were collected at the end of assay 2, air dried, milled, and sent to an external laboratory (CEBAS-CSIC, Murcia, Spain) for chemical analyses. The parameters shown in Table S1 in the supplemental material were used for principal component analysis (PCA).

Soil and rhizosphere sampling. Fifteen-centimeter-deep soil core samples were obtained using a 4-cm-diameter core sampler. Three plants per treatment (named A, B, and C) were randomly selected, three equidistant points around each plant (named R1, R2, and R3) were sampled, and each sample was independently processed and analyzed.

The soil samples were placed in cold storage and transported to the laboratory. Moist field soil was passed through a 2-mm-pore-size sieve, and roots were separated from the bulk soil. The fine avocado roots contained in the sample together with the surrounding adhering soil were defined in the present study as the rhizosphere samples, which included rhizosphere, rhizoplane, and endorhizosphere habitats. The sieved soil that was carefully cleared from the roots was considered the bulk soil sample. Fresh soil and rhizosphere samples were used for culturable microbial population analysis and for community-level Biolog and APIZYM assays (bioMérieux SA, Lyon, France). DNA extraction from the rhizosphere samples was also performed immediately after sample collection, and three subsamples of the bulk soil were stored at −80°C for subsequent DNA extraction.

Microbial isolation and plate counts. For the microbial analysis, the three replicate samples from the same pot were pooled to provide a single composite sample from each plant, and three composite samples per treatment (pots A, B, and C) were analyzed. For the bulk soil analysis, subsamples of 10 g of the bulk soil were suspended in 90 ml of saline solution (0.85% NaCl) with 5 g of sterile gravel (2 to 4 mm in diameter) and mixed at 250 rpm for 30 min on an orbital shaker, which was followed by 20 min of decantation. For the rhizosphere analysis, one gram of the fine roots was homogenized for 2 min in a Stomacher bag with 10 ml of saline solution. In both cases, 10-fold serial dilutions of the supernatant were plated on different selective media.

Selective media were used for the specific isolation of fast-growing heterotrophic bacteria, pseudomonads, sporulating bacteria, actinomycetes, and fungi as described by Larkin and Honeycutt (53). To isolate sporulating bacteria, soil suspensions were pretreated at 80°C for 10 min before plating. Plates were incubated at 23°C for 48 h for the enumeration of fast-growing heterotrophic bacteria, pseudomonads, and sporulating bacteria and for 10 days for actinomycetes and fungi. The average values from triplicate analyses were expressed as CFU per gram of dry soil (oven-dried soil at 105°C for 24 h) or per gram of fresh root.

Sample preparation for the Biolog and APIZYM assays. Soil and rhizosphere suspensions were prepared for the inoculation of community-level physiological profiling and hydrolytic activity assays. The soil suspensions were prepared as follows: 3 g of bulk soil was suspended in 30 ml of saline solution (0.85% NaCl) with 2 g of gravel and horizontally mixed at 250 rpm for 30 min on an orbital shaker. For the rhizosphere suspensions, 2-g portions of fine roots were homogenized for 2 min in a Stomacher bag with 20 ml of saline solution. Every suspension was centrifuged at 50 × *g* for 5 min, and then the supernatant was transferred to a sterile 50-ml tube and centrifuged again at 130 × *g* for 5 min. This low-speed centrifugation has been described as the most efficient method for decreasing the optical density (OD) of a soil suspension by settling the largest soil particles with minimum effect on cell density (54). These suspensions were used for the inoculation of both the Biolog and APIZYM assays.

Community-level physiological profiling assay. The ability of soil microbial communities to use 31 useful carbon sources was assessed using Biolog EcoPlates (Biolog, Inc., Hayward, CA) developed for soil community analysis. Three microtiter plates per treatment and one for each replicate sample (pots A, B, and C) were filled with 150 μl of soil or rhizospheric suspension per well, followed by incubation at 23°C. The utilization rate of the carbon sources was monitored by measuring OD at 590 nm. Color development was analyzed two or three times a day using a microplate reader until the curve of the average well color development (AWCD) reached the saturation point (8 days). The incubation period in which the increase in the AWCD for each plate became maximal was determined and used for the selection of the closest common reading time point for all of the treatments. The OD values were used to compare the physiological profiles, and raw OD data were corrected by subtracting the control OD value (no carbon source provided). Negative values were considered "zero" in subsequent data analyses of net OD. To reduce biases from variations in inoculum density or differences in AWCD, the data were normalized by dividing the net OD of each well by the AWCD (55). A PCA was performed on the normalized data. Substrate richness (S), substrate evenness (E), and metabolic diversity by Shannon index (H) were calculated based on net OD data as described by Zak et al. (56).

Hydrolytic activity assays. Nineteen enzymatic activities were analyzed by using the semiquantitative APIZYM system. APIZYM strips were inoculated with 90 μl of soil or rhizosphere suspension prepared as described below and incubated for 48 h at 23°C. A value ranging from 0 to 5 was assigned according to the colorimetric standard table provided by the manufacturer that relates color intensity with the quantity of hydrolyzed substrate. Three samples per treatment (A, B, and C) and two assay replicates were used to calculate average values.

DNA extraction. DNA was extracted from nine bulk soil samples per treatment (pots A, B, and C and soil core replicates R1, R2, and R3) and nine equivalent rhizosphere samples per treatment. DNA extraction from all soil and rhizosphere samples (0.25 g of soil and 0.3 g of fine roots) was performed using a Power Soil DNA kit (MO-BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol.

PCR amplification of 16S rRNA gene fragments. All DNA samples from soil and rhizosphere were analyzed separately by PCR-DGGE. PCR amplification of the variable region of the bacterial 16S rRNA gene was performed with the universal bacterial primers 341F-GC and 907R described by Muyzer et al. (57, 58). The amplifications were carried out in 50-μl reaction mixtures that consisted of 1 μl of DNA template (ca. 5 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 1 μM concentrations of primers (each), 2.5 U of *Taq* DNA polymerase (all components were from Invitrogen, Carlsbad, CA), and 5% (vol/vol) dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). Touchdown PCR was carried out as described by Muyzer et al. (57, 58).

DGGE profiling. DGGE analyses were conducted using a D-Code Universal detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of PCR product were loaded into the wells of a 6% polyacrylamide gel (acrylamide–*bis*-acrylamide [37.5:1]) containing a gradient of 30 to 60% denaturants (a 100% denaturant concentration was defined as 7 M urea and 40% [vol/vol] deionized formamide). Electrophoresis was performed in 1× Tris-acetate-EDTA buffer at 60°C with a constant voltage of 75 V for 14 h. The PCR products amplified from nine replicates per treatment (pots A, B, and C and soil core replicates R1, R2, and R3) were loaded in the same gel. The lanes on the outsides of the gels were loaded with an unrelated DGGE marker to assist in the normalization and comparison among gels. However, to eliminate potential "gel effects," one replicate from each sample (pots A, B, and C) of the different treatments was loaded in the same gel for a direct comparison among treatments. Gels were stained with ethidium bromide (0.5 μg/ml), destained in distilled water and photographed under UV illumination using a Gel Doc XR+ imaging system (Bio-Rad Laboratories, Inc.).

Analysis of DGGE profiles. Gel images were normalized and analyzed with InfoQuest FP 5.10 software (Bio-Rad Laboratories, Inc.). The Pearson correlation coefficient for each pair of lanes within a gel was calculated as a measure of similarity between the community fingerprints and used to perform cluster analysis by the unweighted pair group method with arithmetic mean (UPGMA). Cophenetic correlation coefficients were calculated to assess the robustness of the assigned clusters. The number and relative intensity of the DGGE bands in each fingerprint were determined using InfoQuest FP software. The number of bands was used as an estimate of the apparent bacterial richness (*S*). Genetic diversity, measured by the Shannon index (*H*), was calculated as $H = -\Sigma \mathrm{pi} \ln(\mathrm{pi})$, where pi is the intensity of each DGGE band divided by the total area of the fingerprint, and the evenness (*E*) was calculated as $E = H/\log(S)$.

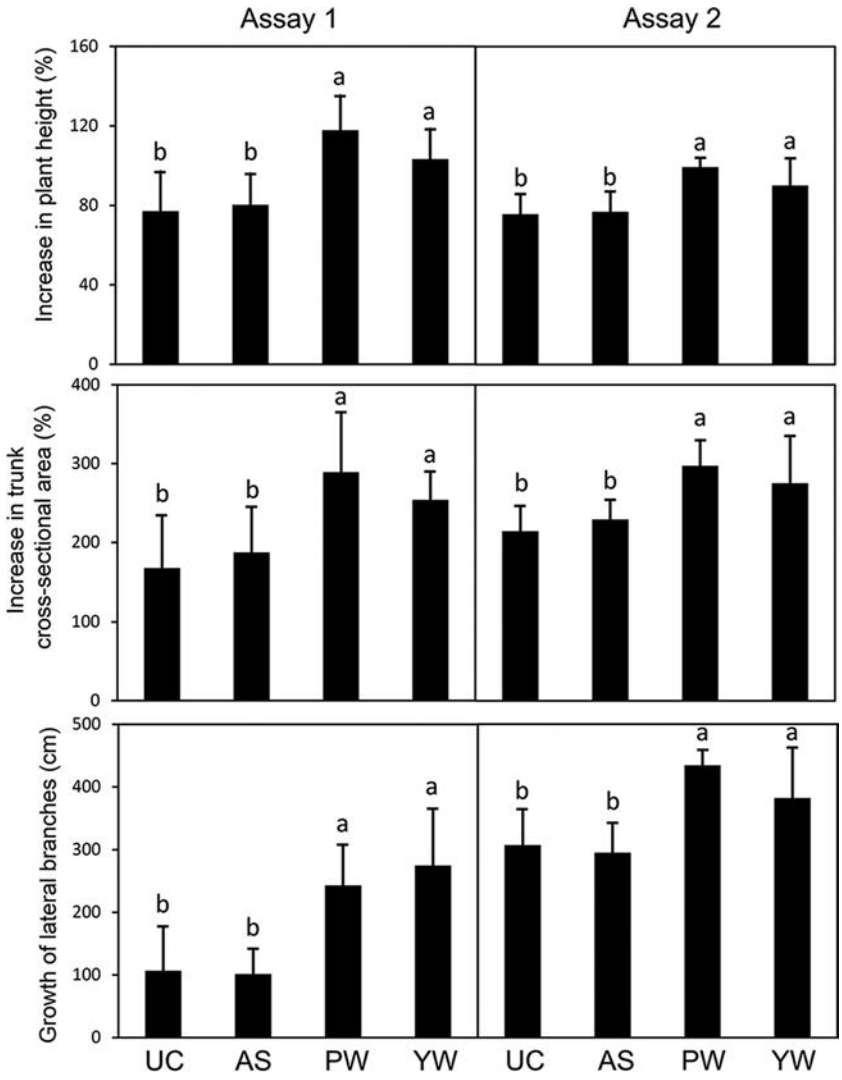


FIG 1 Effect of the organic amendments on avocado plant growth during 9 months of assay. Comparison between amended and unamended control treatment in plant height, trunk cross-sectional area, and growth of lateral branches of noninoculated plants in the two greenhouse experiments. Different letters mean significant differences between treatments (ANOVA, $P < 0.05$). UC, unamended control; AS, almond shells; PW, pruning waste; YW, yard waste.

DGGE band excision, cloning, and sequence analysis. DGGE dominant and/or differentiating bands were excised with a scalpel from the DGGE gels and transferred to 1.5-ml sterile tubes containing 20 μl of sterile MilliQ water, cut into pieces to facilitate DNA elution, and incubated overnight at 4°C. Two microliters of the resulting suspension was used in a PCR to reamplify the excised 16S rRNA gene fragment using the same primers and PCR conditions described for the soil DNA samples. The resulting PCR products were analyzed on a DGGE gel, together with the original community DNA sample, to check their electrophoretic mobility. Excised bands displaying the same melting behavior as the original bands in the community profiles were used as the templates for further PCR amplification with the primers 341F/907R (without a GC clamp). The PCR fragments were ligated into a pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent cells of *Escherichia coli* DH5-α as recommended by the manufacturers. Positive clones were verified by colony PCR and rechecked for comigration with the original band in a new DGGE gel. Selected clones were sent to Macrogen, Inc. (Amsterdam, Netherlands) for sequencing with T7 and SP6 primers. Contigs were assembled with the forward and reverse sequences using the Contig Express software (Vector NTI Advance 10; Invitrogen). The presence of chimeric sequences was detected with DECIPHER's Find Chimeras web tool (59). The resulting sequences were verified for similarity with previously published bacterial 16S rRNA gene sequences in the GenBank database using the BLAST web tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Microcosm assay. Direct inhibition of *R. necatrix* was tested in a microcosm system to check the role of soil microorganisms in the suppressiveness of composted almond shell (AS) amended soil. This microcosm assay was carried out using unamended control (UC) soil, AS-amended soil and several modified versions of these soils. Pasteurized soil (UCp and ASp) was prepared using moist heat treatment at 100°C for 15 min to reduce microbial biomass (60). Four different complemented soils were also prepared to partially recover the microbial community, mixing pasteurized and fresh soil in a 9:1 (wt/wt) proportion (always nine parts of pasteurized soil per one part of fresh soil): UCp+UC, UCp+AS, ASp+UC, and ASp+AS.

Microcosm assays were conducted using a diffusion chamber experimental design adapted from Epstein, 2013 (61). A fungal disk (0.6-cm in

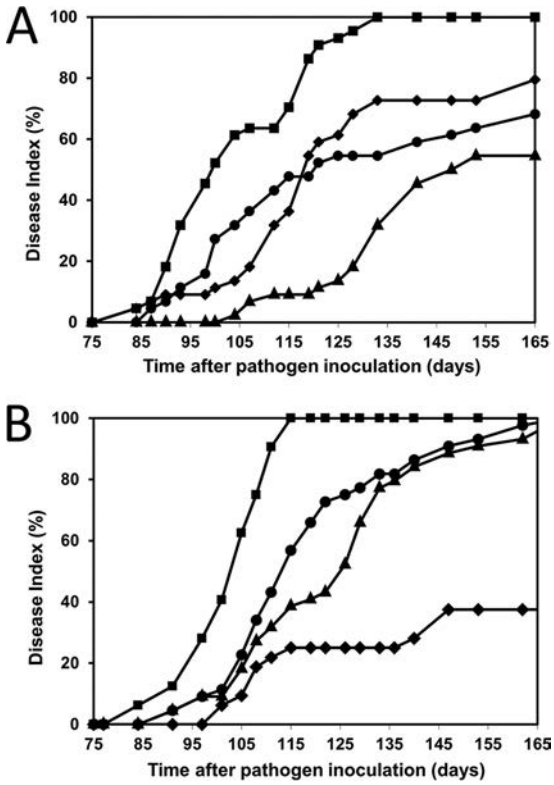


FIG 2 Effect of the organic amendments on avocado white root rot. Time course of the disease index, calculated by evaluation of the aerial symptoms of white root rot in the inoculation assays. (A) Assay 1; (B) assay 2. Symbols: ■, unamended control; ▲, almond shells; ●, pruning waste; ◆, yard waste.

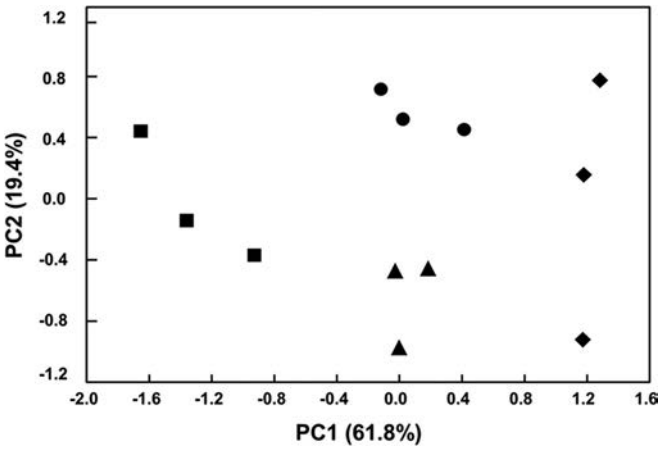


FIG 3 Effect of organic amendments on the chemical properties of the soil. Scatter plot based on PCA of the soil chemical properties of the assay 2. The symbols refer different treatments: ■, unamended control; ▲, almond shells; ●, pruning waste; ◆, yard waste. The data for the chemical composition of the amended soils and the correlation of chemical parameters to ordination axes derived from PCA analysis are available in Tables S1 and S2 in the supplemental material.

diameter) from a 1-week-old culture of *R. necatrix* on potato dextrose agar (PDA) was transferred to a 5-cm-diameter disk of water-agar medium (1%) and placed on top of a nitrocellulose filter (0.45-μm pore size). These multilayer systems were placed on containers on top of the different soils and covered to reduce aerial contamination. A picture of this device is shown in Fig. S3 in supplemental material. Twelve replicate chambers per soil type were incubated for 5 days at 25°C. At the end of the assay, the initial disk area and total growth area of *R. necatrix* were measured using Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc.), and the variation of the area (Δarea) was calculated.

Data analyses. Data distributions were tested for normality using the Shapiro test for normality ($P = 0.05$). For data following a normal distribution, i.e., microbial counts, plant growth parameters, and fungal growth inhibition (microcosms), the differences between treatments were tested using analysis of variance (ANOVA) followed by a Fisher least-significant-difference test ($P = 0.05$). Population density values were $\log_{10}$ transformed before analysis. Disease progression data and diversity indices, which did not show a normal distribution, were compared by nonparametric Mann-Whitney U test ($P = 0.05$). All of these analyses were performed using SPSS software version 15.0 (SPSS, Inc., Chicago, IL). PCA was performed on soil chemical data and Biolog physiological profiles using the demo version of the Multivariate Statistical Package (MVSP, v3.12e; Kovach Computing Service, Anglesey, United Kingdom).

Nucleotide sequence accession numbers. Nucleotide sequence accession numbers were as follows: KF733465 to KF733499 and KF733500 to KF733507 (see Table 4).

RESULTS

Plant growth. In the present study, plants amended with pruning waste (PW) and yard waste (YW) showed a significant increase in growth in comparison to control plants (ANOVA, $P < 0.05$). The effects of YW were more evident in assay 1, where such increase was significant in all of the growth parameters, whereas in assay 2, only trunk cross-sectional area was significantly higher than control (Fig. 1). The treatment with almond shells (AS) showed no significant differences in plant growth from the unamended control (UC).

White root rot progression. The first root rot aerial symptoms appeared ~84 days after inoculation with *R. necatrix* in both of the independent microplot assays. The evolution of the disease index for each treatment with time is shown in Fig. 2. In both of the assays, the unamended control treatment was the first to show aerial symptoms and reach 100% disease index (all of the inoculated plants were dead) at 115 to 135 days postinoculation (assays 1 and 2, respectively). In contrast, the three organic amendments assayed in the present study induced a delay and/or a decrease of white root rot symptoms. The most evident suppressive effect was produced by the AS treatment in the first assay (Fig. 2A) and by the YW treatment in the second assay (Fig. 2B). Statistical comparisons of AUDPC data showed that all of the assayed organic amendments produced a significant reduction (Mann-Whitney U, $P < 0.05$) in white root rot progression compared to the unamended plants.

Chemical soil properties. The chemical characteristics of the amended and unamended soils at the end of the assay (12 months after plant transplantation) are shown in Table S1 in supplemental material. PCA clustered together the three replicate samples from each treatment and separated the different treatments (Fig. 3). This differentiation by treatment indicates both a clear effect of the organic matter on the soil chemical composition and a differential effect depending on the nature of the amendment.

The first principal component (PC1), which explained more than half (61.8%) of the total variance, allowed differentiation between amended and unamended soils and among YW and the other organic treatments. Amended soils, especially the YW treatment, were generally associated with higher levels of total N, total

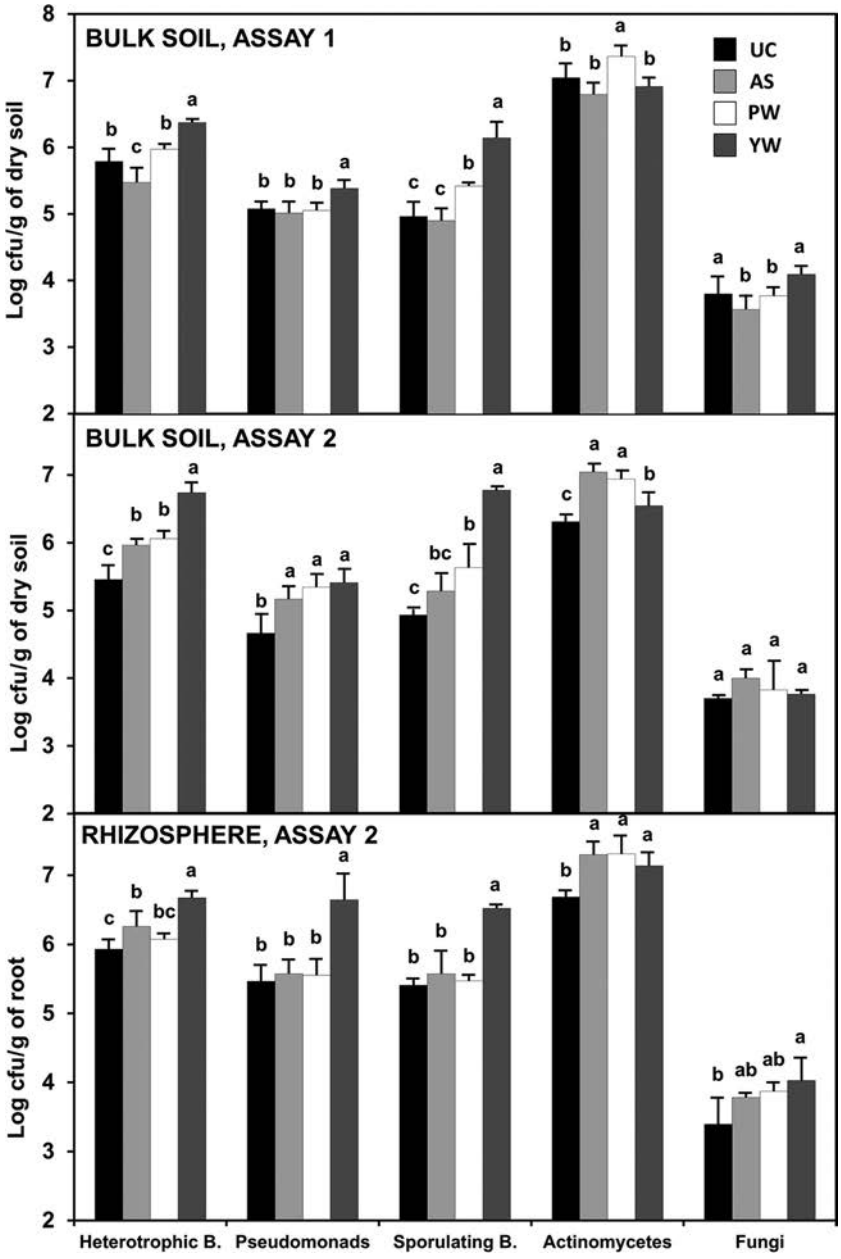


FIG 4 Effect of organic amendments on culturable microorganisms. The population densities of fast-growing heterotrophic bacteria, pseudomonads, sporulating bacteria, actinomycetes, and fungi were assessed by plate counts. Different lowercase letters indicate significant differences (ANOVA, $P < 0.05$). UC, unamended control; AS, almond shells; PW, pruning waste; YW, yard waste.

C, organic C, S, Ca, B, and Mn (significant positive correlations with PC1) and lower levels of Cr, Al, Fe, K, Cd, and Ni (significant negative correlation with PC1) compared to the control soil (see Table S2 in supplemental material). PC2 was closely associated with Zn and Cu values, which explained the differences between the AS and PW treatments and within the YW treatment.

Culturable microbial populations. Microbial counts in the bulk soil and rhizosphere samples showed low levels of fungi (nearly 10^3 to 10^4 CFU per g of dry soil) and higher counts of the different bacterial groups, which has been widely described for soil microbial communities. The actinomycetes generally showed the largest populations (nearly 10^7 CFU per g of dry soil), whereas fast-growing heterotrophic bacterial populations numbered ~10^6 CFU per g of dry soil, indicating a difference greater than one order of magnitude depending on the treatment. The counts of pseudomonads and sporulating bacteria were approximately 10^5 to 10^6 CFU per g of dry soil, also showing significant differences depending on the amendment used.

The YW amendment showed the largest effect, inducing a significant increase (ANOVA, $P < 0.05$) in all of the analyzed bacterial groups in both bulk soil and rhizosphere except for actinomycetes in the soil of assay 1 (Fig. 4). The amendment with PW also increased bacterial numbers, although this effect was lower and mainly restricted to culturable actinomycetes and sporulating bacteria. The AS

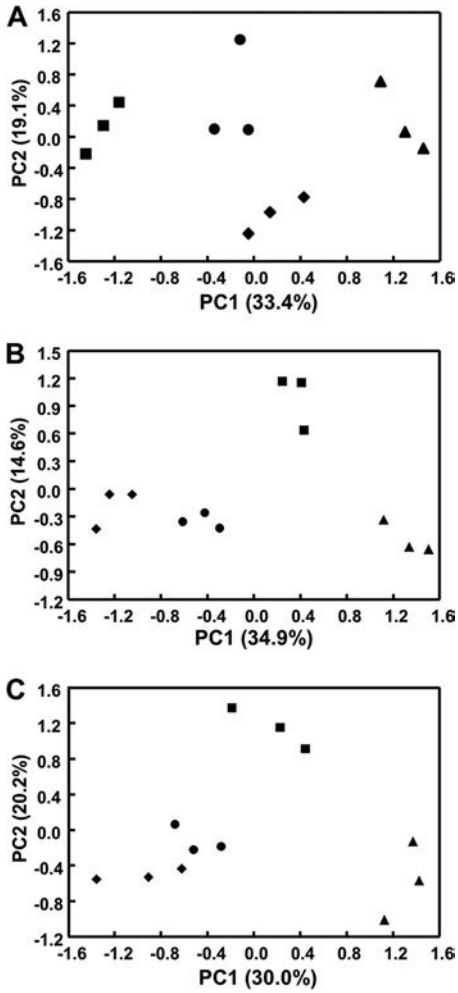


FIG 5 Effect of organic amendments on the metabolic profiles of the microbial community. Scatter plots were prepared based on PCA of normalized OD data of Biolog Ecoplates. (A) Bulk soil of the assay 1; (B) bulk soil of the assay 2; (C) rhizosphere of the assay 2. Symbols: ■, unamended control (UC); ▲, almond shells (AS); ●, pruning waste (PW); ◆, yard waste (YW).

treatment showed the lowest effect on culturable populations and did not affect bacterial populations in assay 1 except for fast-growing heterotrophic bacteria levels, which were even lower than those found in the unamended soil. In assay 2, however, this treatment yielded a slight increase in heterotrophic bacteria and actinomycetes, both in bulk soil and in the rhizosphere (Fig. 4).

Physiological profiles of soil and rhizosphere microbial communities. The initial comparison between Biolog EcoPlate profiles from the soil and rhizosphere samples by PCA clearly differentiated the rhizosphere samples from the bulk soil, but the effect of the treatments was not clear (data not shown). Individual analyses were performed for the bulk soil and rhizosphere samples to detect the effect of the amendments on catabolic profiles. The three replicate samples from the unamended control clustered separately from the amended treatments in both the soil and rhizosphere biplots (Fig. 5). This spatial distribution revealed a clear effect of each different amendment on the metabolic profiles of soil and rhizosphere microbial communities (Fig. 5).

The diversity indices based on Biolog profiles showed slight but statistically significant differences (Mann-Whitney U test, $P < 0.05$) between amended and unamended plants (see Table S3 in supplemental material). In assay 1, the addition of organic amendments increased soil metabolic diversity as determined by Shannon index (H) and richness ($P < 0.05$) compared to the UC, especially for *H*, which was increased by every organic amendment. However, in assay 2, the organic treatments did not show such a clear effect on the diversity indices. The amendment with AS was the only treatment that showed an overall enhancement of metabolic diversity, increasing *H* in both assays in the soil and in the rhizosphere (Mann-Whitney U test, $P < 0.05$) (see Table S3 in the supplemental material).

Extracellular enzyme profiles. The analysis of 19 extracellular hydrolytic enzymes using the APIZYM system revealed a clear influence of the organic amendments on the enzymatic activity profiles of the soil and the rhizosphere. The highest overall activity corresponded with the YW treatment in the soil and rhizosphere, whereas the lowest overall activity always corresponded with the UC (Table 2). Each of the organic treatments induced an increase in the activity of 6 enzymes in bulk soil: acid phosphatase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, and β-glucosidase. In contrast, the only common effect of the addition of organic matter to the enzymatic activities in the rhizosphere was a decrease in alka-

TABLE 2 Enzymatic profiles of soil and rhizosphere of assay 2 based on the hydrolytic activities assessed by the API ZYM system[a]

	Bulk soil				Rhizosphere			
	UC	AS	PW	YW	UC	AS	PW	YW
Phosphatases								
Alkaline phosphatase	2.3	4.7	3.0	5.0	4.3	3.5	3.5	3.5
Acid phosphatase	2.0	4.7	4.0	4.3	4.0	4.5	5.0	4.0
Phosphohydrolase	1.3	3.3	2.0	2.0	4.0	4.0	3.5	3.5
Esterases								
Lipase	0.0	0.3	0.0	1.3	1.7	1.0	3.0	1.0
Esterase Lipase	1.7	2.3	1.3	1.7	2.3	2.5	3.0	2.5
Esterase	1.3	2.3	1.0	1.7	1.3	1.5	2.0	2.0
Amino-peptidases								
Leucine arylamidase	1.0	2.0	2.7	4.3	3.0	2.0	1.5	1.0
Valine arylamidase	1.0	1.0	1.0	1.0	1.3	1.5	0.5	1.5
Cystine arylamidase	0.0	0.0	0.0	0.0	0.0	1.0	0.5	1.0
Proteases								
Trypsin	0.0	0.0	0.0	0.0	0.7	1.0	0.5	3.0
Chymotrypsin	0.0	0.0	0.0	0.0	0.3	1.0	0.0	1.5
Glycosyl-hydrolases								
α-galactosidase	0.0	0.0	0.3	1.0	2.0	2.0	2.0	3.0
β-galactosidase	1.0	2.3	2.3	2.7	2.3	3.0	3.5	3.0
N-acetyl-β-glucosaminidase	1.7	3.3	2.3	2.7	3.0	2.5	3.5	3.0
α-glucosidase	1.3	1.0	3.0	4.0	1.7	1.0	1.5	3.0
β-glucosidase	1.0	3.3	2.3	4.3	2.3	2.5	3.5	3.0
β-glucuronidase	0.0	0.0	0.0	0.0	1.3	2.5	1.0	2.5
α-mannosidase	0.3	0.7	0.0	0.0	1.0	1.0	1.0	2.5
α-fucosidase	0.0	0.7	1.0	1.7	0.3	0.5	1.0	2.0
Total activity	16.0	32.0	26.3	37.7	37.0	38.5	40.0	46.5

[a] Different shadings indicate different intensities of the enzymatic reactions: white, low intensity (0.0 to 1.9); light gray, moderate intensity (2.0 to 3.9); and dark gray, high intensity (4.0 to 5.0). UC, unamended control; AS, almond shells; PW, pruning waste; YW, yard waste.

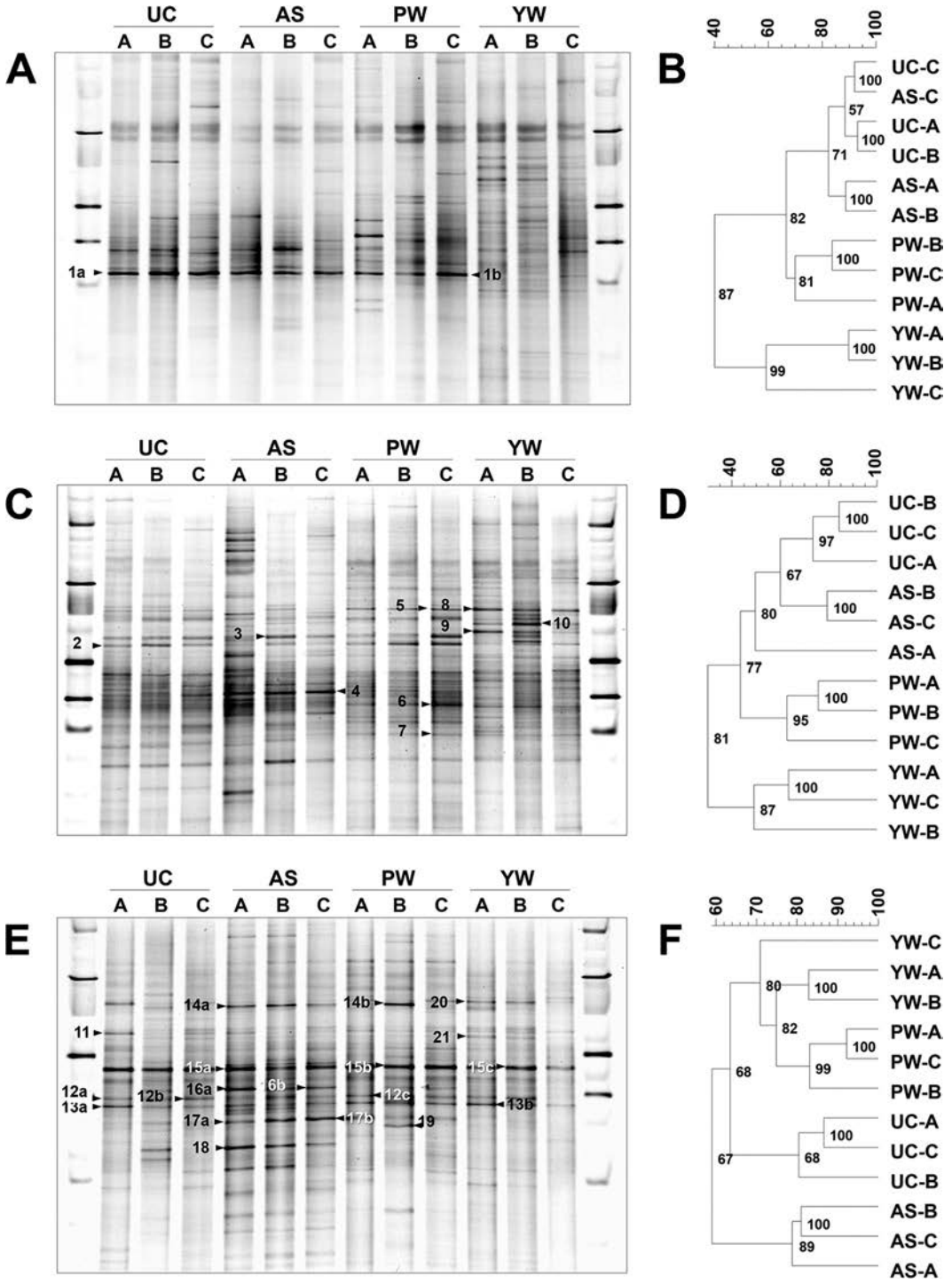


FIG 6 Effect of organic amendments on soil bacterial communities. (A, C, and E) PCR-DGGE fingerprints of bacterial 16S rRNA gene fragments. One replicate from each pot (named A, B, and C) of the different treatments were loaded in the same gel. (B, D, and F) Cluster dendrograms based on Pearson correlation coefficient and UPGMA algorithm showing similarity between 16S DGGE profiles. Numbers at the nodes represent cophenetic correlation values in percent. (A and B) Bulk soil of the assay 1; (C and D) bulk soil of the assay 2; (E and F) rhizosphere of the assay 2. UC, unamended control; AS, almond shells; PW, pruning waste; YW, yard waste. The codes of numbers and letters marked on the DGGE bands from panels A, C, and E correspond to the band codes of 16S rRNA gene sequences shown in Table 4.

line phosphatase activity. Moreover, certain enzymatic activities were specifically enhanced in the soil by the different amendments, whereas their responses to the same amendment in the rhizosphere were more variable (Table 2).

Soil bacterial community analysis by DGGE. In an initial approach that included all of the replicates and used one or two treatments per gel, cluster analysis showed a general treatment-based clustering. Nevertheless, the clustering between treatments

TABLE 3 Genetic diversity indices based on DGGE profiles

DGGE profile	Mean (SD)[a]			
	UC	AS	PW	YW
Soil assay 1				
Shannon	2.53 (0.08)	2.63 (0.20)	2.94 (0.36)	2.83** (0.15)
Richness	17.00 (2.00)	20.00 (3.61)	26.33 (7.02)	22.67** (2.52)
Evenness	0.89 (0.01)	0.88 (0.01)	0.90 (0.04)	0.91 (0.02)
Soil assay 2				
Shannon	2.90 (0.08)	2.95 (0.25)	2.99 (0.08)	3.21** (0.08)
Richness	20.33 (1.15)	24.67 (5.51)	24.00 (2.65)	30.00** (2.65)
Evenness	0.96 (0.01)	0.92* (0.02)	0.94 (0.03)	0.94 (0.01)
Rhizosphere				
Shannon	2.49 (0.12)	2.89** (0.13)	2.46 (0.16)	2.35 (0.10)
Richness	19.00 (1.00)	28.00** (1.00)	18.33 (2.08)	15.33* (1.15)
Evenness	0.85 (0.03)	0.87 (0.03)	0.85 (0.02)	0.86 (0.03)

[a] UC, unamended control; AS, almond shells; PW, pruning waste; YW, yard waste. *, significantly lower than UC ($P < 0.05$); **, significantly higher than UC ($P < 0.05$).

suggested the possibility of a "gel effect" that is commonly related to slight differences in gel quality or running conditions (62, 63). To eliminate this effect, the comparison among treatments was subsequently performed using common gels that contained only one replicate from each sample (pots A, B, and C) of the four assayed treatments. The gels and resulting cluster analyses are shown in Fig. 6. In general, the fingerprints were clustered according to the treatments. The soil samples showed a similar clustering in both of the independent greenhouse experiments. The most similar profiles were displayed by the AS and UC treatments, whereas the YW treatment showed the most dissimilar fingerprints in both of the assays (Fig. 6B and D). In the rhizosphere analysis, the treatment with AS showed the greatest effect on the bacterial community, which was observed both by visual and clustering differences (Fig. 6E and F).

The bacterial genetic diversity based on the number of DGGE bands and their relative intensity was overall higher in the soil of assay 2 than in assay 1. Despite this difference, the treatment YW showed a higher diversity than the other treatments in both assays as revealed by the Shannon and evenness parameters (Mann-Whitney U test, $P = 0.05$), but it did not affect diversity in the rhizosphere (Table 3). However, the treatment with AS increased the bacterial diversity of the rhizosphere but did not affect any of the diversity indices in the soil (Table 3).

Sequence analysis of dominant DGGE bands. In Fig. 6A, C, and E, the bands marked with numbers correspond to the dominant bands that were extracted from the DGGE gels and submitted to cloning and sequencing. Their tentative phylogenetic affiliations are shown in Table 4. The analysis of sequences obtained from the same DGGE band showed occasional heterogeneity within the clones. However, in most cases, they showed equal or very similar phylogenetic affiliations. For every single DGGE band, the number of clones with the same electrophoretic mobility as the original band that was submitted to sequencing and the number of clones displaying identical sequences are shown in Table 4. Only nonidentical sequences were submitted to GenBank under the accession numbers shown in Table 4.

Microcosm assay. The inhibition of *R. necatrix* was tested using eight types of soil to evaluate their suppressive ability and the role of soil microbial communities in disease suppression. The highest inhibition of fungal growth was displayed by the AS-amended fresh soil, with a significantly lower Δarea (ANOVA, $P < 0.05$) than the UC fresh soil and the rest of the soil types (Table 5). Pasteurized soil showed in both cases significantly lower inhibition (ANOVA, $P < 0.05$) than the corresponding fresh soil. Complementation of pasteurized soil with UC fresh soil did not have any significant effect on ASp+UC soil, but it induced a slight but significant recovery of suppressiveness in UCp+UC soil. On the other hand, soil complemented with fresh AS soil showed a clear recovery of soil suppressiveness, showing lower Δarea (ANOVA, $P < 0.05$) than pasteurized soil.

DISCUSSION

The enhancement of soil suppressiveness using organic amendments has been widely described, especially for soilborne diseases (2, 64, 65). However, this effect can be very variable depending on the pathosystem and the environmental conditions, and there are even some examples where the amendment has increased disease incidence (26, 66). Soil organic amendments have been successfully used for the control of *P. cinnamomi* in avocado crops (11, 12, 67), but they have never been tested against the white root rot caused by *R. necatrix* until now. It is therefore interesting that all of the organic amendments tested in the present study showed a suppressive effect against white root rot. The phenomenon of disease suppression has been commonly related to modifications to the soil caused by the organic amendment, including physicochemical properties, microbial populations, and associated processes (27). All of the analyses performed in the present study aimed to understand and identify factors that could account for the suppression of white root rot.

We showed that there was an effect of the organic amendments on the chemical composition of the soil. The gradient in soil nutrient content shown by PCA analysis (PC1 in Fig. 3) corresponded to the gradient of suppressiveness in the same assay (Fig. 2B), suggesting a direct relationship between the nutrient content of the soil and disease suppression. Several soil chemical parameters, which include a high content of nitrogen, carbon, and organic carbon, have been previously correlated to lower disease incidence (26). However, in some cases it is not clear whether this effect was a consequence of the influence of certain soil nutrients on soil microbiota or instead was asso-

TABLE 4 Closest phylogenetic relatives of partial 16S rRNA gene sequences derived from dominant or differentiating DGGE bands

Band code[a]	Sample origin[b]	Treatment[c]	n_{total}[d]	n[e]	NCBI accession no.	Closest phylogenetic relatives		
						Identity/strain	% identity	Accession no.
1a	Soil assay 1	UC	3	2	KF733465	Uncultured *Rhodanobacter* sp. clone AHy52	100	KC502951.1
				1	KF733466	*Rhodanobacter spathiphylli* strain B39	100	NR_042434.1
1b	Soil assay 1	PW	3	3	KF733467	Uncultured *Rhodanobacter* sp. clone AHy5	100	KC502951.1
2	Soil assay 2	UC	1	1	KF733468	*Brevundimonas lenta* strain DS-18	99	NR_044186.1
3	Soil assay 2	AS	2	2	KF733469	Uncultured *Bacteroidetes* bacterium clone BuhD-239	99	FM877553.1
4	Soil assay 2	AS	1	1	KF733470	Uncultured bacterium clone HLLCs310	99	JX100020.1
5	Soil assay 2	PW	1	1	KF733471	*Thiobacter subterraneus* strain C55	91	NR_024834.1
6	Soil assay 2	PW	2	1	KF733472	Uncultured bacterium clone 36	94	FM209350.1
				1	KF733473	Uncultured bacterium clone RamatNadiv01b09	99	JF295396.1
7	Soil assay 2	PW	2	1	KF733474	Uncultured acidobacterium clone GASP-WC2W2_D11	99	EF075273.1
				1	KF733475	"*Candidatus* Solibacter usitatus" strain Ellin6076	93	NR_074351.1
8	Soil assay2	YW	3	2	KF733476	Uncultured *Sphingobacteriales* bacterium clone AMPD3	100	AM936482.1
				1	KF733477	Uncultured betaproteobacterium clone S2-009	99	KF182945.1
9	Soil assay 2	YW	3	3	KF733478	Uncultured *Sphingobacteriales* bacterium clone AMPD3	99	AM936482.1
10	Soil assay 2	YW	2	1	KF733479	Uncultured *Sphingobacteriales* bacterium clone AMPD3	99	AM936482.1
				1	KF733480	Uncultured bacterium DGGE gel band 03_U2 clone 07	99	JX986325.1
11	Rhizos.As2	UC	2	1	KF733481	*Bacteroidetes* bacterium X3-d	99	HM212417.1
				1	KF733482	*Bacteroidetes* bacterium X3-d	99	HM212417.1
12a	Rhizos.As2	UC	3	1	KF733483	*Rubrivivax gelatinosus* strain IL-144	99	NR_074794.1
				1	KF733484	Uncultured bacterium clone sdm16	99	JQ798405.1
				1	KF733485	Uncultured *Burkholderiales* bacterium clone Plot4-E08	99	EU449563.1
12b	Rhizos.As2	UC	5	3	KF733486	Uncultured *Burkholderiales* bacterium clone Plot4-E08	100	EU449563.1
				2	KF733487	Uncultured *Burkholderiales* bacterium clone Plot4-E08	99	EU449563.1
12c	Rhizos.As2	PW	2	2	KF733488	*Rubrivivax gelatinosus* strain IL-144	98	NR_074794.1
13a	Rhizos.As2	UC	1	1	KF733489	*Albidiferax ferrireducens* strain CH1-46	99	KC855480.1
13b	Rhizos.As2	YW	2	2	KF733490	*Albidiferax ferrireducens* strain CH1-46	100	KC855480.1
14a	Rhizos.As2	AS	4	3	KF733491	*Burkholderia* sp. strain K14	100	AJ300687.1
14b	Rhizos.As2	PW	2	2	KF733492	*Burkholderia soli* strain GP25-8	99	NR_043872.1
				1	KF733503	*Cupriavidus oxalaticus* strain NBRC 13593	99	AB680453.1
15a	Rhizos.As2	AS	5	3	KF733493	*Rhizobium leguminosarum* bv. viciae strain 3841	100	NR_103919.1
				1	KF733494	*Rhizobium leguminosarum* bv. viciae strain 3841	99	NR_103919.1
				1	KF733495	*Rhizobium leguminosarum* bv. viciae strain 3841	99	NR_103919.1
15b	Rhizos.As2	PW	2	2	KF733496	*Rhizobium leguminosarum* bv. viciae strain 3841	100	NR_103919.1
15c	Rhizos.As2	YW	1	1	KF733497	*Rhizobium leguminosarum* bv. viciae strain 3841	100	NR_103919.1
16a	Rhizos.As2	AS	1	1	KF733498	Uncultured bacterium clone HLLCs310	100	JX100020.1
16b	Rhizos.As2	AS	1	1	KF733499	Uncultured bacterium clone HLLCs310	100	JX100020.1
17a	Rhizos.As2	AS	2	2	KF733500	*Frateuria aurantia* strain DSM 6220	99	NR_074107.1
17b	Rhizos.As2	AS	2	2	KF733501	*Frateuria aurantia* strain DSM 6220	99	NR_074107.1
18	Rhizos.As2	AS	2	2	KF733502	*Burkholderia tuberum* strain STM678	98	NR_027554.1
19	Rhizos.As2	PW	1	1	KF733504	*Rhodanobacter thiooxydans* strain LCS2	99	NR_041565.1
20	Rhizos.As2	YW	3	2	KF733505	Uncultured betaproteobacterium clone GASP-WC1W2_B07	99	EF074724.1
				1	KF733506	Uncultured *Sphingobacteriales* bacterium clone AMPD3	100	AM935103.1
21	Rhizos.As2	YW	2	2	KF733507	Uncultured *Bacteroidetes* bacterium clone L1-7	99	JF703503.1

[a] Band codes refer to the DGGE bands marked in Fig. 8.
[b] Origin of the samples for sample types and assays: soil assay 1, soil assay 2, or rhizosphere assay 2 (Rhizos.As2).
[c] UC, unamended control; AS, almond shells; PW, pruning waste; YW, yard waste.
[d] n_{total}, total number of clones with the same electrophoretic mobility of the original band that had been subjected to sequencing.
[e] n, number of clones sharing identical 16S rRNA gene sequences.

ciated with an enhancement of plant growth and vigor. In fact, mulching of avocado crops in field studies increases fruit production through improving the growth and health of surface feeder roots (8, 9). These healthier roots could also potentially be more resistant to attack by soilborne pathogens such as *P. cinnamomi* or *R. necatrix*. In the present study, we did not find any evidence of this type of effect; on the contrary, we demonstrated with a microcosm assay that bulk soil amended with AS has a direct inhibitory effect on the growth of the fungus *R. necatrix*, so the plant is not necessarily involved in the mechanism of disease suppression. This experiment helped to clarify several details related to suppression mechanisms. For example, the reduction of the inhibitory effect from partial sterilization of the soil demonstrated that the suppressiveness of AS has a microbial origin. In fact, in the literature it is widely assumed that the mechanisms of disease suppression are mainly biological, whereas abiotic traits of the soil could only indirectly modulate the efficacy of suppression through their effect on the plant and/or on the pathogenic process (22, 68). Soil sup-

TABLE 5 Microcosm assay to evaluate the role of microbial community in suppressive soils[a]

Soil sample	Δarea	SD
UC	38.76[B]	1.95
UCp	46.03[A]	0.97
UCp+UC	38.81[B]	2.23
UCp+AS	34.75[C]	0.71
AS	28.75[D]	1.87
ASp	39.52[B]	1.98
ASp+UC	37.16[B]	1.86
ASp+AS	32.41[C]	0.36

[a] The average of growth area variation (Δarea) from different types of soils and their standard deviations are shown. UC, unamended control; AS, almond shells amended soil; UCp, pasteurized control soil; ASp, pasteurized amended soil. Complemented soils in a 9:1 (wt/wt) proportion were prepared by mixing 9 parts of pasteurized soil with 1 part of fresh soil in the following combinations: UCp+UC, UCp+AS, ASp+UC, and ASp+AS. Superscript capital letters indicate significant differences between treatments (ANOVA, $P = 0.05$)

pressiveness has been correlated with quantitative and qualitative changes in soil microbiota, including increases in microbial biomass (30, 31) and microbial diversity (23, 32) and changes on microbial community composition (33, 34). Many studies have focused only on phylogenetic traits, but microbial activity and functional diversity might be as important as phylogenetic traits when studying disease suppression (24, 27).

The APIZYM system has been demonstrated to be a fast but suitable method for assessing hydrolytic activities in soil. The enzymatic activity assays are not based on microbial growth, so they might reflect, at least theoretically, the *in situ* community function more closely than culture-based methods (69). In the present study, six hydrolytic enzymes were substantially enhanced in bulk soil by the addition of organic matter regardless of the nature of the amendment used. Some of these enzymes are key enzymes in primary biogeochemical cycles, frequently used as indicators of soil functioning, and their activation in soil has been widely related to the addition of vegetal composts and amendments (70–73). Especially interesting is the enhancement of *N*-acetyl-β-glucosaminidase activity, which is one of the enzymes involved in chitin degradation. Chitinolytic activity has been related to the control of several fungal diseases by single biocontrol microorganisms and to the use of compost (38, 74, 75). The sum of total hydrolytic activity in bulk soil showed that soil with higher activity was also more efficient at reducing white root rot symptoms. In the case of YW, the strong increase in soil and rhizospheric hydrolytic activity is probably related to the large increase in bacterial population levels caused by this amendment. In addition to their influence on enzymatic activities, an increase in bacterial populations (especially those of total heterotrophic bacteria and sporulating bacteria) was described by Bonanomi et al. (27) as a good predictor of the suppressive potential of an organic amendment. In this instance, an enhancement of bacterial populations might be related to the suppressive effect of the YW treatment, but it is likely not involved in the suppressive effect of AS, which barely affected microbial population size.

Previous studies have also demonstrated that organic amendments influence the composition and diversity of soil bacterial communities in avocado orchards (20). In the present study, the DGGE results provided further evidence for the ability of organic amendments to affect microbial communities, both in the soil and the rhizosphere of avocado plants. Once again YW showed the strongest effect on bulk soil, causing the most important changes in bacterial community composition and a significant increase of soil bacterial diversity. Unlike the YW treatment, the amendment with AS, which also showed high suppressive ability, scarcely affected the bacterial community composition in bulk soil as analyzed by DGGE. However, the addition of AS especially affected microbial communities in the rhizosphere, where bacterial diversity was increased and several populations were specifically enhanced. Attempts to identify these populations by sequencing of DGGE bands often leads to uncertain phylogenetic affiliations, and most soil-living bacteria have only been detected before by molecular methods and are therefore barely known. We successfully identified here two interesting bacterial populations that were enhanced in the rhizosphere of AS-amended plants. The species *Burkholderia tuberum* is part of the group of nonpathogenic *Burkholderia* species associated with plants, which include biocontrol agents and N-fixing nodulating bacteria and are considered to be potentially beneficial (76). *Frateuria aurantia* is a naturally occurring beneficial proteobacterium widely known for its ability to solubilize fixed potassium into an exchangeable form, making it assumable by plants, and commercial formulations of this bacterium are approved for use in organic agriculture (77).

As mentioned before, the microcosm assay revealed that the suppressiveness of AS has a microbial origin. Therefore, it proves that the effect of AS on soil microorganisms is responsible for turning the conducive UC soil into a suppressive soil. Thus, the AS is necessarily affecting the bulk soil microbiota, even if the PCR-DGGE method used in the present study was not able to detect large differences in bacterial community composition. The effect of AS on bulk soil was, however, detected by the analysis of soil physiological profiles and enzymatic activities. In fact, AS was the only amendment that showed a significant and consistent increase in potential metabolic diversity (based on Biolog data) both in the rhizosphere and in the bulk soil in the two plant assays. In the case of AS, the restoration of suppressiveness by complementation of sterile soil with a small proportion of fresh amended soil suggests a mechanism of specific suppression wherein some specific populations and activities should be mainly responsible for the disease control phenomenon (60). This type of mechanism agrees with the more subtle effects on soil microbiota caused by the addition of AS in the present study, where it did not show a clear enhancement of microbial populations or activities, but it induced specific structural and physiological changes.

All of the results observed here suggest that the amendments with AS and PW owe their disease suppression capacity to different mechanisms. The addition of YW increased the overall soil enzymatic activities, bacterial population levels, and diversity and caused important changes in soil bacterial community composition. These effects suggest a general increase in soil health and functioning that is presumably behind the suppressiveness of YW-amended soil through a mechanism of general suppression. This type of disease suppression is related to an overall boost of microbial communities and activities, and no specific population can be pointed to as mainly for responsible disease control (60). General disease suppression is frequently enhanced by organic matter input and has been related to increased soil fertility (2), which is in concordance with the increase in nutrient content and plant growth caused by the addition of YW. However, the suppressiveness triggered by AS seems to be related to a mechanism of specific

suppression caused by more subtle structural and physiological changes that could trigger the stimulation of specific microbial activities.

Based on the comparative analysis performed here, a single mechanism cannot be identified as the causal factor of disease suppression. In fact, the suppressive effect should be attributable not to a single mechanism but to a combination of causes, especially in the case of YW. Nevertheless, all of the organic amendments assayed in the present study were able to suppress the white root rot caused by *R. necatrix* to some extent, despite the differential nature of their suppressive effects. These amendments should be considered an effective agricultural practice for the control of white root rot in organic avocado crops.

ACKNOWLEDGMENTS

We thank José M. Hermoso, Jorge González, and Emilio Guirado for assistance in suppressive assay design and management. We thank Irene Linares and Juan A. Torés for their assistance during various parts of the project. We are especially grateful to José M. Farré for helpful ideas and discussions, which were essential for this project.

This study was supported by Plan Nacional I+D+I from Ministerio de Ciencia e Innovación (MICINN; AGL08-05453-C02-01 and AGL11-30354-C02-01), cofinanced by FEDER funds (EU). N.B. was supported by a Ph.D. fellowship from the FPU program of MICINN, and C.V. was supported by a Ph.D. fellowship from the FPI program of MICINN.

REFERENCES

1. **Fonte SJ, Yeboah E, Ofori P, Quansah GW, Vanlauwe B, Six J.** 2009. Fertilizer and residue quality effects on organic matter stabilization in soil aggregates. Soil Sci Soc Am J **73:**961–966. http://dx.doi.org/10.2136/sssaj2008.0204.
2. **Bailey KL, Lazarovits G.** 2003. Suppressing soil-borne diseases with residue management and organic amendments. Soil Tillage Res **72:**169–180. http://dx.doi.org/10.1016/S0167-1987(03)00086-2.
3. **Lazarovits G.** 2001. Management of soil-borne plant pathogens with organic soil amendments: a disease control strategy salvaged from the past. Can J Plant Pathol **23:**1–7. http://dx.doi.org/10.1080/07060660109506901.
4. **Bonanomi G, Antignani V, Pane C, Scala F.** 2007. Suppression of soilborne fungal diseases with organic amendments. J Plant Pathol **89:**311–324.
5. **Zhou XG, Everts KL.** 2004. Suppression of *Fusarium* wilt of watermelon by soil amendment with hairy vetch. Plant Dis **88:**1357–1365. http://dx.doi.org/10.1094/PDIS.2004.88.12.1357.
6. **Delgado MM, Martin JV, Miralles R, León-Cófreces C, García MC.** 2010. Phytotoxicity of uncomposted and composted poultry manure. Afr J Plant Sci **4:**154–162.
7. **Cannel RQ.** 1981. Potentials and problems of simplified cultivation and conservation tillage. Outlook Agric **10:**379–384.
8. **Wolstenholme BN, Moore-Gordon CS, Cowan AK.** 1997. Orchard mulching effects on avocado fruiting, p 119–130. *In* Cutting JG (ed), Proceedings from the Australian Avocado Growers' Association Conference '97: Searching for Quality, Rotorua, New Zealand, 23 to 26 September 1997.
9. **Moore-Gordon C, Cowan AK, Wolstenholme BN.** 1997. Mulching of avocado orchards to increase Hass yield and fruit size and boost financial rewards a three season summary of research findings, p 46–49. *In* South African Avocado Growers' Association Yearbook, vol 20. SAAGA, Tzaneen, South Africa.
10. **Hermoso JM, Torres MD, Farré JM.** 2011. Effects of organic materials on adult Hass trees, p 5. Proceedings of the Seventh World Avocado Congress, Cairns, Australia, 5 to 9 September 2011.
11. **Bender GS, Casale WL, Rahimian M.** 1992. Use of worm-composted sludge as a soil amendment for avocados in *Phytophthora*-infested soil, p 143. *In* Proceedings of the Second World Avocado Congress, Orange, CA, 21 to 26 April 1991.
12. **Downer AJ, Menge JA, Pond E.** 2001. Association of cellulytic enzyme activities in eucalyptus mulches with biological control of *Phytophthora cinnamomi*. Phytopathology **91:**847–855. http://dx.doi.org/10.1094/PHYTO.2001.91.9.847.
13. **Pliego C, López-Herrera C, Ramos C, Cazorla FM.** 2012. Developing tools to unravel the biological secrets of *Rosellinia necatrix*, an emergent threat to woody crops. Mol Plant Pathol **13:**226–239. http://dx.doi.org/10.1111/j.1364-3703.2011.00753.x.
14. **Wallis PD, Haynes RJ, Hunter CH, Morris CD.** 2010. Effect of land use and management on soil bacterial biodiversity as measured by PCR-DGGE. Appl Soil Ecol **46:**147–150. http://dx.doi.org/10.1016/j.apsoil.2010.06.006.
15. **Stark CH, Condron LM, O'Callaghan M, Stewart A, Di HJ.** 2008. Differences in soil enzyme activities, microbial community structure and short-term nitrogen mineralisation resulting from farm management history and organic matter amendments. Soil Biol Biochem **40:**1352–1363. http://dx.doi.org/10.1016/j.soilbio.2007.09.025.
16. **Saison C, Degrange V, Oliver R, Millard P, Commeaux C, Montange D, Le Roux X.** 2006. Alteration and resilience of the soil microbial community following compost amendment: effects of compost level and compost-borne microbial community. Environ Microbiol **8:**247–257. http://dx.doi.org/10.1111/j.1462-2920.2005.00892.x.
17. **Ceja-Navarro JA, Rivera-Orduña FN, Patiño-Zúñiga L, Vila-Sanjurjo A, Crossa J, Govaerts B, Dendooven L.** 2010. Phylogenetic and multivariate analyses to determine the effects of different tillage and residue management practices on soil bacterial communities. Appl Environ Microbiol **76:**3685–3691. http://dx.doi.org/10.1128/AEM.02726-09.
18. **Gomez E, Ferreras L, Toresani S.** 2006. Soil bacterial functional diversity as influenced by organic amendment application. Bioresour Technol **97:**1484–1489. http://dx.doi.org/10.1016/j.biortech.2005.06.021.
19. **Gomiero T, Pimentel D, Paoletti MG.** 2011. Environmental impact of different agricultural management practices: conventional versus organic agriculture. Crit Rev Plant Sci **30:**95–124. http://dx.doi.org/10.1080/07352689.2011.554355.
20. **Bonilla N, Cazorla FM, Martínez-Alonso M, Hermoso JM, González-Fernández J, Gaju N, Landa BB, de Vicente A.** 2012. Organic amendments and land management affect bacterial community composition, diversity, and biomass in avocado crop soils. Plant Soil **357:**215–226. http://dx.doi.org/10.1007/s11104-012-1155-1.
21. **Yogev A, Raviv M, Hadar Y, Cohen R, Katan J.** 2006. Plant waste-based composts suppressive to diseases caused by pathogenic *Fusarium oxysporum*. Eur J Plant Pathol **116:**267–278. http://dx.doi.org/10.1007/s10658-006-9058-8.
22. **Hoitink HAJ, Fahy PC.** 1986. Basis for the control of soilborne plant pathogens with composts. Annu Rev Phytopathol **24:**93–114. http://dx.doi.org/10.1146/annurev.py.24.090186.000521.
23. **van Elsas JD, Garbeva P, Salles J.** 2002. Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soilborne plant pathogens. Biodegradation **13:**29–40. http://dx.doi.org/10.1023/A:1016393915414.
24. **Bonilla N, Gutiérrez-Barranquero J, Vicente A, Cazorla F.** 2012. Enhancing soil quality and plant health through suppressive organic amendments. Diversity **4:**475–491. http://dx.doi.org/10.3390/d4040475.
25. **Mazzola M.** 2004. Assessment and menagement of soil microbial community structure for disease suppression. Annu Rev Phytopathol **42:**35–59. http://dx.doi.org/10.1146/annurev.phyto.42.040803.140408.
26. **Janvier C, Villeneuve F, Alabouvette C, Edel-Hermann V, Mateille T, Steinberg C.** 2007. Soil health through soil disease suppression: which strategy from descriptors to indicators? Soil Biol Biochem **39:**1–23. http://dx.doi.org/10.1016/j.soilbio.2006.07.001.
27. **Bonanomi G, Antignani V, Capodilupo M, Scala F.** 2010. Identifying the characteristics of organic soil amendments that suppress soilborne plant diseases. Soil Biol Biochem **42:**136–144. http://dx.doi.org/10.1016/j.soilbio.2009.10.012.
28. **Duffy BK, Ownley BH, Weller DM.** 1997. Soil chemical and physical properties associated with suppression of take-all of wheat by *Trichoderma koningii*. Phytopathology **87:**1118–1124. http://dx.doi.org/10.1094/PHYTO.1997.87.11.1118.
29. **Tenuta M, Lazarovits G.** 2002. Ammonia and nitrous acid from nitrogenous amendments kill microsclerotia of *Verticillium dahliae*. Phytopathology **92:**255–264. http://dx.doi.org/10.1094/PHYTO.2002.92.3.255.
30. **van Os GJ, van Ginkel JH.** 2001. Suppression of *Pythium* root rot in bulbous *Iris* in relation to biomass and activity of the soil microflora. Soil Biol Biochem **33:**1447–1454. http://dx.doi.org/10.1016/S0038-0717(01)00053-0.
31. **Bulluck LR, Ristaino JB.** 2002. Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of

processing tomatoes. Phytopathology **92:**181–189. http://dx.doi.org/10.1094/PHYTO.2002.92.2.181.
32. **Garbeva P, Postma J, Van Veen JA, Van Elsas JD.** 2006. Effect of above-ground plant species on soil microbial community structure and its impact on suppression of *Rhizoctonia solani* AG3. Environ Microbiol **8:**233–246. http://dx.doi.org/10.1111/j.1462-2920.2005.00888.x.
33. **Cohen MF, Yamasaki H, Mazzola M.** 2005. Brassica napus seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of *Rhizoctonia* root rot. Soil Biol Biochem **37:**1215–1227. http://dx.doi.org/10.1016/j.soilbio.2004.11.027.
34. **Pérez-Piqueres A, Edel-Hermann V, Alabouvette C, Steinberg C.** 2006. Response of soil microbial communities to compost amendments. Soil Biol Biochem **38:**460–470. http://dx.doi.org/10.1016/j.soilbio.2005.05.025.
35. **Erhart E, Burian K, Hartl W, Stich K.** 1999. Suppression of *Pythium ultimum* by biowaste composts in relation to compost microbial miomass, activity and content of phenolic compounds. J Phytopathol **147:**299–305.
36. **Pane C, Spaccini R, Piccolo A, Scala F, Bonanomi G.** 2011. Compost amendments enhance peat suppressiveness to *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotinia minor*. Biol Control **56:**115–124. http://dx.doi.org/10.1016/j.biocontrol.2010.10.002.
37. **Rasmussen PH, Knudsen IMB, Elmholt S, Jensen DF.** 2002. Relationship between soil cellulolytic activity and suppression of seedling blight of barley in arable soils. Appl Soil Ecol **19:**91–96. http://dx.doi.org/10.1016/S0929-1393(01)00177-9.
38. **Chae DH, Jin RD, Hwangbo H, Kim YH, Kim YW, Park RD, Krishnan HB, Kim KY.** 2006. Control of late blight (*Phytophthora capsici*) in pepper plant with a compost containing multitude of chitinase-producing bacteria. BioControl **51:**339–351.
39. **Alabouvette C, Backhouse D, Steinberg C, Donovan NJ, Edel-Hermann V, Burgess LW.** 2004. Microbial diversity in soil: effects on crop health, p 121–138. *In* Schjonning P, Elmholt S, Christensen BT (ed), Managing soil quality: challenges in modern agriculture. CAB International, Wallingford, United Kingdom.
40. **Steinberg C, Edel-Hermann V, Alabouvette C, Lemanceau P.** 2007. Soil suppressiveness to plant diseases, p 455–478. *In* van Elsas JD, Jansson JC, Trevors JT (ed), Modern soil microbiology, 2nd ed. CRC Press, Inc, Boca Raton, FL.
41. **Nybroe O, Brandt KK, Nicolaisen MH, Sørensen J.** 2007. Methods to detect and quantify bacteria in soil, p 283–316. *In* van Elsas JD, Jansson JK, Trevors JT (ed), Modern soil microbiology, 2nd ed. CRC Press, Inc, Boca Raton, FL.
42. **Marzorati M, Wittebolle L, Boon N, Daffonchio D, Verstraete W.** 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ Microbiol **10:**1571–1581. http://dx.doi.org/10.1111/j.1462-2920.2008.01572.x.
43. **Liu B, Gumpertz ML, Hu S, Ristaino JB.** 2007. Long-term effects of organic and synthetic soil fertility amendments on soil microbial communities and the development of southern blight. Soil Biol Biochem **39:**2302–2316. http://dx.doi.org/10.1016/j.soilbio.2007.04.001.
44. **Smalla K, Wieland G, Buchner A, Zock A, Parzy J, Kaiser S, Roskot N, Heuer H, Berg G.** 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. Appl Environ Microbiol **67:**4742–4751. http://dx.doi.org/10.1128/AEM.67.10.4742-4751.2001.
45. **Garbeva P, van Veen JA, van Elsas JD.** 2004. Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu Rev Phytopathol **42:**243–270. http://dx.doi.org/10.1146/annurev.phyto.42.012604.135455.
46. **Costa R, Gomes NCM, Krögerrecklenfort E, Opelt K, Berg G, Smalla K.** 2007. *Pseudomonas* community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses. Environ Microbiol **9:**2260–2273. http://dx.doi.org/10.1111/j.1462-2920.2007.01340.x.
47. **Girvan MS, Bullimore J, Ball AS, Pretty JN, Osborn AM.** 2004. Responses of active bacterial and fungal communities in soils under winter wheat to different fertilizer and pesticide regimens. Appl Environ Microbiol **70:**2692–2701. http://dx.doi.org/10.1128/AEM.70.5.2692-2701.2004.
48. **Torsvik V, Øvreås L.** 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol **5:**240–245. http://dx.doi.org/10.1016/S1369-5274(02)00324-7.
49. **Kennedy AC, Smith KL.** 1995. Soil microbial diversity and the sustainability of agricultural soil. Plant Soil **170:**75–87. http://dx.doi.org/10.1007/BF02183056.
50. **Sztejnberg A, Madar Z.** 1980. Host range of *Dematophora necatrix*, the cause of white root rot disease in fruit trees. Plant Dis **64:**662–664. http://dx.doi.org/10.1094/PD-64-662.
51. **Ruano-Rosa D, del Moral-Navarrete L, Lopez-Herrera C.** 2010. Selection of *Trichoderma* spp. isolates antagonistic to *Rosellinia necatrix*. Span J Agric Res **8:**1084–1097. http://dx.doi.org/10.5424/sjar/2010084-1403.
52. **Cazorla FM, Duckett SB, Bergstrom ET, Noreen S, Odijk R, Lugtenberg BJJ, Thomas-Oates JE, Bloemberg GV.** 2006. Biocontrol of avocado *Dematophora* root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-hexyl 5-propyl resorcinol. Mol Plant-Microbe Interact **19:**418–428. http://dx.doi.org/10.1094/MPMI-19-0418.
53. **Larkin RP, Honeycutt CW.** 2006. Effects of different 3-year cropping systems on soil microbial communities and rhizoctonia diseases of potato. Phytopathology **96:**68–79. http://dx.doi.org/10.1094/PHYTO-96-0068.
54. **Calbrix R, Laval K, Barray S.** 2005. Analysis of the potential functional diversity of the bacterial community in soil: a reproducible procedure using sole-carbon-source utilization profiles. Eur J Soil Biol **41:**11–20. http://dx.doi.org/10.1016/j.ejsobi.2005.02.004.
55. **Garland JL, Mills AL.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl Environ Microbiol **57:**2351–2359.
56. **Zak JC, Willig MR, Moorhead DL, Wildman HG.** 1994. Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem **26:**1101–1108. http://dx.doi.org/10.1016/0038-0717(94)90131-7.
57. **Muyzer G, de Waal EC, Uitterlinden AG.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol **59:**695–700.
58. **Muyzer G, Hottentrager S, Teske A, Wawer C.** 1996. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA: a new molecular approach to analyze the genetic diversity of mixed microbial communities, p 3.4.4.1–3.4.4.22. *In* Akkermans ADL, van Elsas JD, de Bruijn FJ (ed), Molecular microbial ecology manual. Kluwer Academic Publishing, Dordrecht, The Netherlands.
59. **Wright ES, Yilmaz LS, Noguera DR.** 2012. DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Appl Environ Microbiol **78:**717–725. http://dx.doi.org/10.1128/AEM.06516-11.
60. **Weller DM, Raaijmakers JM, McSpadden BB, Thomashow LS.** 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annu Rev Phytopathol **40:**309–348. http://dx.doi.org/10.1146/annurev.phyto.40.030402.110010.
61. **Epstein SS.** 2013. The phenomenon of microbial uncultivability. Curr Opin Microbiol **16:**636–642. http://dx.doi.org/10.1016/j.mib.2013.08.003.
62. **Moeseneder MM, Arrieta JM, Muyzer G, Winter C, Herndl GJ.** 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl Environ Microbiol **65:**3518–3525.
63. **Marschner P.** 2007. Soil microbial community structure and function assessed by FAME, PLFA, and DGGE: advantages and limitations, p 181–200. *In* Varma A, Oelmüller R (ed), Advanced techniques in soil microbiology, vol 11. Springer-Verlag, Heidelberg, Germany.
64. **Lazarovits G, Tenuta M, Conn K.** 2001. Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops. Australas Plant Pathol **30:**111–117. http://dx.doi.org/10.1071/AP01009.
65. **van Elsas JD, Postma J.** 2007. Suppression of soil-borne phytopathogens by compost, p 201–214. *In* Diaz LF, de Bertoldi M, Bidlingmaier W, Stentiford E (ed), Compost science and technology, vol 8. Elsevier, Amsterdam, The Netherlands.
66. **Termorshuizen AJ, van Rijn E, van der Gaag DJ, Alabouvette C, Chen Y, Lagerlöf J, Malandrakis AA, Paplomatas EJ, Rämert B, Ryckeboer J, Steinberg C, Zmora-Nahum S.** 2006. Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. Soil Biol Biochem **38:**2461–2477. http://dx.doi.org/10.1016/j.soilbio.2006.03.002.
67. **Menge J, McDonald V.** 2004. Management of soil microorganisms for the control of *Phytophthora* root rot. Phytopathology **94:**S125.
68. **Griffin DE.** 2012. Managing abiotic factors of compost to increase soilborne disease suppression. J Nat Resour Life Sci Educ **41:**31–34. http://dx.doi.org/10.4195/jnrlse.2012.0003se.
69. **Tiquia SM.** 2005. Microbiological parameters as indicators of compost

maturity. J Appl Microbiol **99:**816–828. http://dx.doi.org/10.1111/j.1365-2672.2005.02673.x.

70. **Marcote I, Hernández T, Garcıa C, Polo A.** 2001. Influence of one or two successive annual applications of organic fertilizers on the enzyme activity of a soil under barley cultivation. Bioresour Technol **79:**147–154. http://dx.doi.org/10.1016/S0960-8524(01)00048-7.
71. **Zhang YL, Chen LJ, Sun CX, Wu ZJ, Chen ZH, Dong GH.** 2010. Soil hydrolase activities and kinetic properties as affected by wheat cropping systems of northeastern China. Plant Soil Environ **56:**526–532.
72. **Ros M, Pascual JA, Garcia C, Hernandez MT, Insam H.** 2006. Hydrolase activities, microbial biomass and bacterial community in a soil after long-term amendment with different composts. Soil Biol Biochem **38:**3443–3452. http://dx.doi.org/10.1016/j.soilbio.2006.05.017.
73. **Ramos ME, Benítez E, García PA, Robles AB.** 2010. Cover crops under different managements versus frequent tillage in almond orchards in semiarid conditions: effects on soil quality. Appl Soil Ecol **44:**6–14. http://dx.doi.org/10.1016/j.apsoil.2009.08.005.
74. **Ahmed AS, Ezziyyani M, Pérez-Sánchez C, Candela ME.** 2003. Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. Eur J Plant Pathol **109:**633–637. http://dx.doi.org/10.1023/A:1024734216814.
75. **Viterbo A, Ramot O, Chernin L, Chet I.** 2002. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. Antonie Van Leeuwenhoek **81:**549–556. http://dx.doi.org/10.1023/A:1020553421740.
76. **Suárez-Moreno ZR, Caballero-Mellado J, Coutinho BG, Mendonça-Previato L, James E, Venturi V.** 2012. Common features of environmental and potentially beneficial plant-associated *Burkholderia*. Microb Ecol **63:**249–266. http://dx.doi.org/10.1007/s00248-011-9929-1.
77. **Sahoo R, Bhardwaj D, Tuteja N.** 2013. Biofertilizers: a sustainable eco-friendly agricultural approach to crop improvement, p 403–432. *In* Tuteja N, Singh Gill S (ed), Plant acclimation to environmental stress. Springer, New York, NY.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 22 January 2016
doi: 10.3389/fmicb.2016.00004

Microbial Profiling of a Suppressiveness-Induced Agricultural Soil Amended with Composted Almond Shells

*Carmen Vida, Nuria Bonilla, Antonio de Vicente and Francisco M. Cazorla **

Departamento de Microbiología, Facultad de Ciencias, Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga, Consejo Superior de Investigaciones Científicas, Málaga, Spain

This study focused on the microbial profile present in an agricultural soil that becomes suppressive after the application of composted almond shells (AS) as organic amendments. For this purpose, we analyzed the functions and composition of the complex communities present in an experimental orchard of 40-year-old avocado trees, many of them historically amended with composted almond shells. The role of microbes in the suppression of *Rosellinia necatrix*, the causative agent of avocado white root rot, was determined after heat-treatment and complementation experiments with different types of soil. Bacterial and fungal profiles obtained from natural soil samples based on the 16S rRNA gene and ITS sequencing revealed slight differences among the amended (AS) and unamended (CT) soils. When the soil was under the influence of composted almond shells as organic amendments, an increase in *Proteobacteria* and *Ascomycota* groups was observed, as well as a reduction in *Acidobacteria* and *Mortierellales*. Complementary to these findings, functional analysis by GeoChip 4.6 confirmed these subtle differences, mainly present in the relative abundance of genes involved in the carbon cycle. Interestingly, a group of specific probes included in the "soil benefit" category was present only in AS-amended soils, corresponding to specific microorganisms previously described as potential biocontrol agents, such as *Pseudomonas* spp., *Burkholderia* spp., or *Actinobacteria*. Considering the results of both analyses, we determined that AS-amendments to the soil led to an increase in some orders of *Gammaproteobacteria*, *Betaproteobacteria,* and *Dothideomycetes*, as well as a reduction in the abundance of *Xylariales* fungi (where *R. necatrix* is allocated). The combination of microbial action and substrate properties of suppressiveness are discussed.

Keywords: soil, amendment, almond shells, microbial profiling, suppressiveness

OPEN ACCESS

Edited by:
Aurelio Ciancio, Istituto per la Protezione Sostenibile delle Piante del CNR, Italy

Reviewed by:
Raffaella Balestrini, Consiglio Nazionale delle Ricerche, Italy
Giancarlo Roccuzzo, Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Italy

****Correspondence:***
Francisco M. Cazorla
cazorla@uma.es

Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *22 October 2015*
Accepted: *05 January 2016*
Published: *22 January 2016*

Citation:
Vida C, Bonilla N, de Vicente A and Cazorla FM (2016) Microbial Profiling of a Suppressiveness-Induced Agricultural Soil Amended with Composted Almond Shells. Front. Microbiol. 7:4. doi: 10.3389/fmicb.2016.00004

INTRODUCTION

The enhancement of soil suppressiveness using organic amendments has been widely described, especially for soil-borne diseases (Lazarovits et al., 2001; Bailey and Lazarovits, 2003; van Elsas and Postma, 2007; Bonilla et al., 2012b; Pane et al., 2013). However, this effect can be extremely variable depending on the pathosystem and the environmental conditions, and there are even some examples of the amendment application increasing disease incidence (Termorshuizen et al., 2006;

Janvier et al., 2007). The soils that become suppressive soils provide an environment in which plant disease development is reduced, even in the presence of a virulent pathogen and a susceptible host (Hadar and Papadopoulou, 2012). This phenomenon could be induced as a direct result of the activity of microorganism consortia that are naturally established on soil after application of the amendment (Weller et al., 2002).

As such, understanding the diversity, composition, structure, function and interactions of microbial communities is crucial to gain insight into the basis for suppressiveness mediated by this organic amendment (Janvier et al., 2007). Approaches for studying microbial communities in the soil are complex. Thus, employing genomic approaches to understand which changes occur in soil could be a good alternative strategy to decipher the profiling of soil microbiota (Garbeva et al., 2004).

The use of genomic techniques rely on PCR amplification of the conserved and variable regions of the microbial genome, commonly 16S ribosomal RNA (rRNA) for bacteria and 18S rRNA or internal transcribed sequences (ITS) for fungi, allowing for direct sequencing of these PCR amplicons using different high-throughput next-generation sequencing methods. Each group of PCR amplicons that shares a similar or identical variable region is considered an "operational taxonomic unit" (OTU) and is assumed to be equivalent to a microbial species or genus. The analysis of OTUs provide information about the phylogenetic diversity of the soil microbial community (van Elsas et al., 2007, 2008; Hirsch et al., 2013; Koyama et al., 2014).

Moreover, complementary techniques have arisen, such as microarrays, which have considerable potential in environmental microbial ecology, providing novel insights into how environmental factors affect microbial communities in various habitats (Hazen et al., 2010; He et al., 2012; Bai et al., 2013; Zhang et al., 2013; Tu et al., 2014). The GeoChip microarray is a comprehensive functional gene array (FGA) targeting hundreds to thousands of different gene families that play important roles in various biogeochemical processes, enabling researchers to comprehensively analyse the functional diversity, composition, and structure of microbial communities in various environments. It is a powerful FGA-based technology that can be used to survey the functional diversity, composition, structure, metabolic potential/activity, and dynamics of microbial communities, and then link them with ecosystem processes and functions (Xie et al., 2011; Xue et al., 2013; Cong et al., 2015).

Our research interest is focused on the avocado (*Persea americana* Mill.), for which southern Spain is one of the most relevant zones in the Mediterranean area for this crop. In this part of the world, one of the most limiting soilborne diseases affecting avocado trees is white root rot, caused by the fungus *R. necatrix* Prill. White root rot is considered to be an emergent threat to many woody crop plants worldwide (Pliego et al., 2009, 2012).

The role of soil microorganisms in the plant protection have been broadly reported. Thus, different microbes can contribute to the biocontrol of avocado white root rot using different weaponry such as antagonism (*Pseudomonas chlororaphis* PCL1606 or *Bacillus subtilis* PCL1608; Cazorla et al., 2006, 2007), competition for niches and nutrients (Calderón et al., 2014), or induction of systemic resistance or predation (*Trichoderma* spp.; Ruano-Rosa and López-Herrera, 2009). These microorganisms can act as single or combined with other biocontrol agents against *R. necatrix* (Ruano-Rosa et al., 2014). Other studies have reported the positive effect of the application of arbuscular mycorrhizal fungi to soil and the biocontrol activity on avocado (Hass and Menge, 1990; González-Cortés et al., 2012).

During the past decades, several approaches have been implemented to achieve an integrated management of *R. necatrix,* including physical, chemical and biological control approaches (López-Herrera et al., 1998; López-Herrera and Zea-Bonilla, 2007; González-Sánchez et al., 2013). All of these approaches seem to be effective at the experimental level, and some of them have been proven to be effective under certain conditions. However, at the same time, traditional strategies of land management have improved, and some of these strategies could be considered useful approaches to fight against diseases in avocado management, thus increasing the weaponry available against white root rot (Bonilla et al., 2012b).

One of these approaches is the use of organic amendments or mulches, which have produced beneficial effects for plants, including increasing health and yields in avocado crops (Moore-Gordon et al., 1997; Wolstenholme et al., 1997; Hermoso et al., 2011). It has been previously shown that the application of such organic matter to avocado agricultural soil can affect soil physicochemical properties and microbial communities (Bonilla et al., 2012a; López et al., 2014). Additionally, organic amendments could play a critical role in global biochemical cycles (Bonanomi et al., 2014) and could cause different effects, such as the improvement of soil fertility and the enhancement of natural suppressiveness of the soil against several phytopathogens (Cretoiu et al., 2013). Several organic amendments have shown an obvious suppressive effect against another important avocado soil-borne phytopathogen, *Phytophthora cinnamomi* (Bender et al., 1992; Downer et al., 2001).

In a previous study, it was shown that different organic matter applied as a mulch to the avocado crop exhibited suppressive effects against white root rot (Bonilla et al., 2015). Composted almond shells were one type of organic matter tested. The application of composted almond shells as a mulch led to an enhancement of the bacterial composition and activities of the soil communities in relation to the observed suppressiveness (Bonilla et al., 2015).

The objective of the present study was to gain insight into the microbial profiling present in the amended soils showing suppressive ability against the avocado soil-borne phytopathogen *R. necatrix*. The use of different microbial approaches should uncover the microbial communities potentially involved in the suppressive phenotype.

MATERIALS AND METHODS

Field of Study

Soil samples were obtained from an avocado crop field (cv. *Hass* avocado trees grafted onto cv. *Topa-Topa* seedling rootstocks) located at the Experimental Station "La Mayora"

(IHSM-UMA-CSIC, Málaga, Spain) on the coast of the Malaga Province (SE Spain). This experimental field of 2.5 km^2 (36°75′N, 4°04′O) contains 195 40-year-old avocado trees planted at 8 × 8 m. Selected avocado trees were grouped in pairs to facilitate their management. Sixteen pairs of trees were under ecological management (massive application of composted almond shells in 2002, 2007, and 2012), and another 16 pairs of trees were under conventional management (addition of mineral nutrients twice per year, as well as the application of herbicides and pesticides when necessary, López et al., 2014) and without any organic amendment.

Soil Sampling

Natural field soil samples allocated underneath of avocado trees unamended (CT) or amended with composted almond shells (AS) were taken to perform the different experiments. Soil samples were collected in April 2013, November 2013 and April 2014. Composite soil samples were taken from four different groups of paired trees with (AS) or without (CT) organic amendment and were randomly selected from throughout the avocado orchard. To obtain a composite soil sample, two sampling distal points at 1.5 m around the trunk base for each tree of a pair of trees under the same treatment were selected; the upper layer of compost was carefully removed, and 5–10 kg of soil samples (15 cm depth) were collected per pair of trees and merged. Samples were placed in cold storage and transported to the laboratory. Samples of each type of soil were sieved through a 20 mm mesh and immediately used for physicochemical and suppressiveness experiments. To perform DNA extractions, three soil samples (1 g each) from composite soil samples per each pair of trees were sieved again (2 mm diameter) and processed independently. The remaining unused soil samples were stored at −80°C.

Physicochemical Analysis of Soil Samples

Physicochemical analysis of both types of soil samples were performed at Laboratorio Caisur S.L. (Granada, Spain) using standardized methodologies. Four samples from each composite field soil sample (AS and CT) were analyzed independently.

Soil Processing

To test the potential role of soil microorganisms in suppressiveness, we prepared three types of processed soils using different treatments: Field soils (raw soils), heat-treated soils, and complemented soils (**Table 1**). We applied a moist heat treatment to the field soil samples as previously described (Weller et al., 2002), with slight modifications. Briefly, the heat treatment consisted of heating the soil in high moisture conditions at 100°C for 20 min in an autoclave. The soil was allowed to recover at 4°C overnight. Then, we performed a second treatment step, heating the soil at 100°C for 10 min in high moisture conditions. After allowing it to cool, the soil was ready to be used (**Figure 1**). Complemented soils were prepared with the purpose of observing the partial recovery of the microbial characteristics of the natural soil (Weller et al., 2002). The complemented soil consisted of heat-treated soil mixed with natural raw field soil in a 9:1 (w/w) ratio (**Table 1**).

TABLE 1 | Types of processed agricultural soils used in this study.

Soil source	Treatment code	Details of processed soils
Amended with composted almond shells	AS	Natural field soil amended with composted almond shells mulching
	ASt	AS heat-treated soil
	ASc	ASt complemented with AS in 9:1 (w/w) ratio
	ASt+CT	ASt complemented with CT in 9:1 (w/w) ratio
Unamended and under conventional management	CT	Natural field soil unamended and under conventional management
	CTt	CT heat-treated soil
	CTc	CTt complemented with CT in 9:1 (w/w) ratio
	CTt+AS	CTt complemented with AS in 9:1 (w/w) ratio

*A scheme of the processing is described in **Figure 1**.*

To evaluate changes in the culturable microbiota fraction during different times of the soil sample processing, counts of cultivable colony forming units (CFUs) of bacteria and fungi per gram of soil were performed. For this, 2 g samples of soil obtained at the different key times during the process were suspended in 20 ml of sterile saline solution (0.85% NaCl) with 0.5 g of sterile gravel and mixed at 150 rpm for 30 min on an orbital shaker at room temperature. Ten-fold serial dilutions of the obtained suspensions were plated on Luria Bertani (LB) agar with 100 mg of cycloheximide per liter, to analyse the heterotrophic bacteria group, and on potato dextrose agar (PDA) with 50 mg of chlortetracycline and 1 ml of tergitol NP-10 (Sigma) per liter (Bonilla et al., 2012a).

Suppressiveness Assays

Suppressiveness assays against white root rot caused by the virulent strain *R. necatrix* CH53 (López-Herrera and Zea-Bonilla, 2007) were conducted using two different susceptible pathosystems, avocado (Cazorla et al., 2006) and wheat (*Triticum aestivum*). The *R. necatrix* inoculum was produced on wheat seeds (Freeman et al., 1986). The seeds were soaked for 12 h in 250-ml Erlenmeyer flasks filled with distilled water. The flasks were autoclaved after excess water had been drained off. After sterilization, fungal disks of a 1-week-old culture of *R. necatrix* grown on PDA were placed aseptically in each flask. Flasks were incubated at 25°C for 2–3 weeks and were shaken every 2–3 days to avoid clustering of the seeds.

Avocado/*R. necatrix* Test System

Six-month-old commercial avocado plants were obtained from Brokaw nurseries (Brokaw España, S.L., Vélez-Málaga, Spain). The roots from the avocado plants were disinfected by immersion in 0.1% NaOCl for 20 min and then washed twice (20 min) with sterile distilled water. Then, avocado plants were placed into square plastic pots (10.5 × 10.5 × 10.5 cm) containing 0.64 L of the

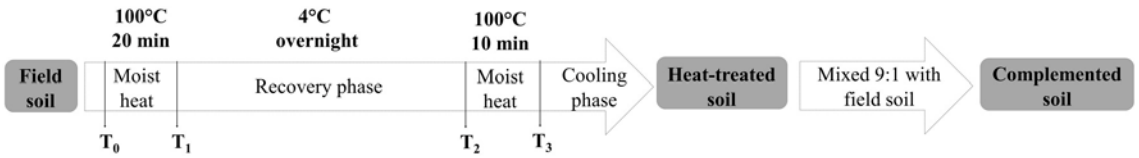


FIGURE 1 | Processing scheme of the soil heat-treatment and complementation used in this study for the agricultural field soil samples. The same procedure was followed for both unamended soil and soil amended with composted almond shells. $T_{0–3}$ indicates sampling points to perform bacterial and fungal plate counts.

sieved CT and AS types of soils. Fungal infection with *R. necatrix* was performed using wheat grains (4 holes of 2 cm depth were made per pot, 3 infected wheat grains were placed per hole) as previously described (Freeman et al., 1986). Non-infected plants were used as controls. Three sets of 15 avocado plants were tested per type of soil. The plants were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms of avocado white root rot were recorded on a scale of 0–3, and a disease index (DI) was calculated after 5 weeks using the previously described formula (Cazorla et al., 2006).

Wheat/*R. necatrix* Test System

Wheat seeds were disinfected by immersion in 0.05% NaOCl for 10 min, washed and then placed in darkness between pieces of moist filter paper in a growth chamber for 2–3 days at 25°C to induce germination. Then, germinated seedlings were disinfected again by immersion in 0.1% NaOCl for 20 min and washed (20 min) with sterile distilled water. Seedlings were placed into plastic seedling trays (5 cm diameter × 5.5 cm) containing 0.08 L of different types of soils and either infected with *R. necatrix* using wheat grains (three grains per slot) or not infected to be used as controls. Three sets of 50 wheat seedlings were tested per type of soil. The seedlings were grown in a chamber at 25°C with 70% relative humidity and 16 h of daylight and were watered twice per week. Aerial symptoms were evaluated, and the disease index percentage was calculated as previously described for the avocado/*R. necatrix* system (Cazorla et al., 2006). Disease index percentage was recorded after evaluation of symptoms, with values ranging between 0 (healthy plant), 1 (yellowing stem base), 2 (drying stem base), and 3 (dead plant). The number of diseased seedlings was determined 7 weeks after beginning the assay, and the disease index was calculated as previously described (Cazorla et al., 2006).

Soil DNA Extraction

Soil DNA extraction was performed using 1.0 g of soil samples and a PowerSoil® DNA Isolation Kit (MOBIO Laboratories, Inc, Carlsbad, CA, USA). DNA was extracted from three independent soil samples per pair of trees for amended and unamended soil (AS and CT) and checked for quality. To test the DNA quality we performed a DNA digestion using the restriction enzyme *Eco*RI (New England BioLabs®, Inc., Ipswich, MA, UK) and PCR amplification of the variable region of the bacterial 16S rDNA with the universal bacterial primers 341F and 907R as described by Muyzer et al. (2004). Digestion and PCR products were analyzed for size by agarose gel electrophoresis and ethidium bromide staining. Suitable samples were mixed and DNA quantity and quality ($A_{260}/A_{230} > 1.8$ and $A_{260}/A_{280} > 1.7$) were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Three independent DNA extractions were performed per each pair of trees, and then merged to create a composite DNA sample. Three of these composite DNA extractions were independently analyzed for each type of field soil (AS and CT). DNA was stored at −20°C for further analyses.

Analysis of 16S rRNA and its Gene Sequence

Two composite DNA samples from each soil type were sent for sequencing by STAB VIDA (NGS Laboratories, Caparica, Portugal) and sent to ChunLab (Seoul, Korea) to obtain the microbial DNA sequences of the 16S rRNA gene and ITS hypervariable regions. Sequences were analyzed using QIIME software (Caporaso et al., 2010) and CLcommunity™ software (ChunLab). Sequences of a length less than 200 nt were excluded from the analysis. The data were filtered for noisy sequences, checked for the presence of chimeras, and binned into OTUs (Peiffer et al., 2013) at the 97% sequence similarity level. A representative sequence of each OTU was taxonomically classified. The relative abundance of microbial clades at different taxonomic levels was calculated as the average value from two independent analyses and was used to perform the comparative distribution analysis.

Geochip Analysis

Three of the composite samples of purified test DNA (800 ng per sample) from the two different types of soils studied (AS and CT) were sent to Glomics Inc (Norman, Oklahoma) for the sequencing analysis (Tu et al., 2014). Briefly, after the hybridization steps, the arrays were washing, dried and then scanned. The images obtained were analyzed by NimbleScan software (Roche NimbleGen Inc., Madison, WI) using the gridding file containing GeoChip 4.6 probes and NimbleGen control probes to determine the intensity of each spot and to identify low quality spots, which were removed prior to statistical analysis (probe spots with coefficient of variance > 0.8 were removed). Extracted data were then loaded into the GeoChip data analysis pipeline at the Institute for Environmental Genomics (Microarray Data Manager, http://ieg.ou.edu/microarray/; Liang et al., 2010; Deng and Zhou, 2013). First, the average signal

intensity of the common oligo reference standard (CORS) was calculated for each array, and the maximum average value was applied to normalize the signal intensity of samples in each array. Second, the sum of the signal intensity of the samples was calculated for each array, and the maximum sum value was applied to normalize the signal intensity of all of the spots on an array, which produced a normalized value for each spot in each array. Spots were scored as positive based on a floating signal-to-noise ratio [SNR = (signal mean–background mean)/background standard deviation] so that hyperthermophile control probes accounted for 5% of positive probes. Spots that were detected in less than two samples were also removed. Before statistical analysis, logarithmic transformation was carried out for the remaining spots, and the signals of all spots were transformed into relative abundances (the sum of the number of hybridized probes for each gene category or gene function between the number of total detected probes).

Data processing was used for further analyses. Genes that overlapped between treatments (AS and CT) were calculated by dividing the number of overlapped genes between the treatments by the number of all genes detected in both treatments. Gene function diversity was calculated using the Shannon-Weiner index (H', alpha diversity) and Simpson's index (1/D, beta diversity). We performed a detrended correspondence analysis (DCA) to measure the differences of community functional gene structure between treatments. For comparing the different gene function communities, a hierarchical clustering analysis using Bray-Curtis distances was also performed. To analyse the unique detected probes in the AS samples, we performed a Venn diagram analysis using an on-line tool (http://bioinfogp.cnb.csic.es/tools/venny/). Previously, we prepared two databases by selecting genes (probes) that hybridize exclusively in each type of soil and compared them. This website provided us with a list of 2766 AS unique detected sequences from suppressive soil, which were selected to perform specific comparative analysis.

Statistical Methods

For suppressive analysis, the data were statistically analyzed using an analysis of variance (Sokal and Rohlf, 1986), followed by Fisher's least significant difference test ($P = 0.05$) using SPSS 22 software (SPSS Inc., Chicago). For GeoChip 4.6 analysis, significant differences in relative abundances of the microbial gene diversity between different soils were analyzed by an unpaired Student's *t*-test. A significance level of $P < 0.1$ was adopted for all comparisons. Based on the standard error, the 95% confident interval for each response variable was obtained and the significant differences between the soils were estimated.

RESULTS

Characteristics of Avocado Field Soils

The soil samples were taken from the same avocado orchard but from trees under different soil management (AS-amended or unamended). Soil characteristics of the experimental avocado field revealed sandy-loam textures for the amended (AS) and unamended (CT) soils. The pH was not substantially different among these samples and ranged from 7.20 to 7.55 (nearly neutral pH). Some macro- and micro-nutrients, such as potassium, iron and manganese, were also increased in the AS-amended soil (data not shown).

White Root Rot Suppressiveness Assay

Suppressiveness assays against white root rot were performed using the avocado/*R. necatrix* and the wheat/*R. necatrix* experimental plant test systems. AS-amended and unamended avocado agricultural soils, after different experimental heat treatments an complementantions were used (**Figure 1**; **Table 1**).

Bacterial and fungal counts of AS-amended and CT soil were very similar, with values of 6.5 and 6.6 $\log_{10}$ bacterial cfu/g, respectively, and 5.0 and 5.1 $\log_{10}$ fungal cfu/g, respectively. After the heat treatment of the soil, bacterial counts decreased and stabilized, without any further changes after a second heat treatment in any type of soil (**Table 2**). There were no differences in the results obtained for fungal count (**Table 2**).

For avocado/*Rosellinia* test system, the disease incidence was evaluated after 5 weeks and at the end of the assay, and the disease index (DI) was calculated (**Figure 2A**). In these studies, AS field soil samples displayed better suppressive ability than CT field soil samples. Plants growing in the presence of AS-amended soil samples displayed a significantly lower DI than plants cultivated in the presence of CT soil samples at the end of the experiment (**Figure 2A**). The disease suppressiveness activity was reduced when AS soil samples were heat-treated (ASt) but showed no changes in CTt soil. Moreover, suppressiveness was complemented by soils ASc and CTt+AS, when incorporating AS soil samples. Complemented soil ASt+CT and CTc did not have a disease-suppressive ability, with levels resembling those for the heat-treated unamended soil (**Figure 2A**).

For the wheat/*R. necatrix* plant test system, disease incidence was tested 7 weeks after inoculation when the disease index (DI) was calculated (**Figure 2B**). Similar to the results shown by the avocado/*R. necatrix* test system, the AS-amended soil exhibited better suppressive ability than CT soil. The suppressiveness phenotype was significantly lost in heat-treated soils (ASt and CTt) and was partially recovered when we used amended field soil to complement (ASc and CTt+AS). The soils complemented with unamended soil, CTt and ASt+CT, had a disease-suppressive ability similar to that of heat-treated unamended soil (**Figure 2B**).

TABLE 2 | Plate counts of total heterotrophic bacteria and fungi during the soil heat-treatment of the unamended and amended with composted almond shells.

Plate counts of	Soil source sample	Sampling points during the heat-treatment process			
		T_0	T_1	T_2	T_3
Heterotrophic bacteria	AS	6.5 ± 0.48	5.9 ± 0.76	6.0 ± 0.42	5.9 ± 0.59
	CT	6.6 ± 0.30	5.9 ± 0.64	5.9 ± 0.30	5.7 ± 0.64
Heterotrophic fungi	AS	5.0 ± 0.90	4.7 ± 0.67	4.9 ± 0.57	4.7 ± 0.60
	CT	5.1 ± 0.98	4.9 ± 0.55	5.0 ± 0.67	4.8 ± 0.87

$T_{0–3}$ indicates sampling points used along the process. Microbial counts data are presented as log10 cfu/g soil ± standard deviation.

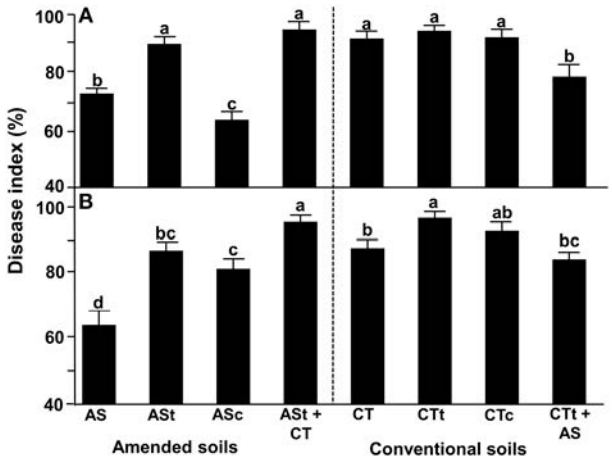


FIGURE 2 | Suppressiveness assays using the avocado/*R. necatrix* (A) and wheat/*R. necatrix* (B) test systems. AS, agricultural field soil amended with composted almond shells; ASt, AS heat-treated soil; ASc, ASt complemented with AS in 9:1 (w/w) ratio; ASt+CT, ASt complemented with CT in 9:1 (w/w) ratio; CT, Agricultural field soil under conventional management; CTt, CT heat-treated soil; CTc, CTt complemented with CT in 9:1 (w/w) ratio; CTt+AS, CTt complemented with AS in 9:1 (w/w) ratio. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test ($P = 0.05$). Values of bars with different letters indications denote a statistically significant difference.

Characterization of the Soil Microbial Community Based on 16S rRNA Gene and its Sequencing

DNA profiling approaches and the sequencing of 16S rRNA and the ITS variable regions of extracted and mixed DNA revealed the relative abundances of microbial clades at different taxonomic levels. However, only the most abundant OTUs were quantified with a level of precision sufficient to perform the comparative distribution analysis due to the high level of OTU richness.

In both samples, *Archaea* were found in a very low relative abundance (<0.1%). Thus, the bacterial 16S rRNA gene sequences allowed us to identify 33 different representative phyla in AS soil samples and 26 phyla in CT soil samples, from which 5 and 7 phyla comprise more than 1% of the community in AS and CT, respectively (**Figure 3**).

In AS soil samples, the 5 most abundant phyla (above 89% of relative abundance) were *Proteobacteria* (50.08%), *Acidobacteria* (22.64%), *Bacteroidetes* (8.05%), *Planctomycetes* (4.27%), and *Actinobacteria* (4.09%). In contrast, the analysis of CT soil samples revealed that the most abundant (representing above 95%) phyla were *Proteobacteria* (45.48%), *Acidobacteria* (27.39%), *Bacteroidetes* (8.79%), *Planctomycetes* (60.99%), *Actinobacteria* (3.19%), *Nitrospirae* (1.70%), and *Gemmatimonadetes* (1.63%).

At the class level, the AS soils presented a high abundance of uncultured bacteria from the groups of *Acidobacteria* (EU686603, 18.44%), *Gammaproteobacteria* (17.85%), *Alphaproteobacteria* (15.28%), and *Betaproteobacteria* (11.4%) (**Figure 3**). In CT soil samples, the class analysis resulted in a similar representation of class abundance, including uncultured bacteria EU686603 (22.99%), *Alphaproteobacteria* (17.7%), and *Gammaproteobacteria* (10.7%).

In both soil samples, the phylum *Proteobacteria* is the most abundant (50.08 and 45.48%). Differences in this group have been shown between the two soil samples. In general, diversity is higher in AS soil samples that exhibit a predominance of the classes *Gammaproteobacteria* (36%) and *Alphaproteobacteria* (30%) and a low percentage of *Deltaproteobacteria*. In CT soil samples, a clear predominance of *Alphaproteobacteria* can be observed (39%). Remarkably, we observed an increase in AS soil samples (almost 2x) of the orders *Steroidobacter* (28%) and *Burkholderiales* (13%) and the decrease of *Rhodospirales* (from 18% in CT to 8% in AS) (Figure S1A).

We observed 76 different classes in AS soil samples and 65 classes in CT soil samples. We detected 24 and 13 specific bacterial classes in AS and CT, respectively, and a slightly higher richness in AS samples (Figure S2A).

The analysis of ITS sequences to reveal the abundance of eukaryotic microbes allowed us to identify a high abundance of fungal microbes. Eukaryotic microbes different from fungi ranged from 7.97% (AS) to 9.52 (CT). Among the fungi detected, the unclassified fungi comprises 8.04% (AS) and 4.28% (CT), and those below 1% represent 2.9% in CT soil samples and 3.4% in AS soil samples.

The most abundant fungal groups (approximately 70%) that are in both soil samples are of the phyla *Ascomycota* and *Basidiomycota* and of the group *Mortierellales*. In AS soil samples, an increase in the relative abundance of *Ascomycota* can be observed (**Figure 4**), (35.37% in CT and 45.79% in AS), as well as a reduction in the group of *Mortierellales* (18.37 in CT and 9.92% in AS).

The analysis of the most abundant group of microorganisms (*Ascomycota*) revealed that in AS soil samples an increase of the class of *Dothideomycetes* (from 40% in CT to 54% in AS) was observed. Additionally, a reduction of the class of *Sordariomycetes* (from 38% in CT to 29% in AS) was observed. Also of note in reference to fungal order in AS soil samples, a huge increase of *Pleosporales* (from 16% in CT to 48% in AS) was observed. Remarkably, one of the fungal order that decreased in AS soil samples was the order *Xylariales* (from 8% in CT to 3% in AS), where the pathogen *R. necatrix* is allocated (Figure S1B).

We observed 39 different classes in AS soil and 50 classes in CT soil. We detected 7 and 18 specific bacterial classes in AS and CT soil, respectively, and observed a slightly higher richness in CT samples (Figure S2B).

GeoChip Analysis in Soil Samples

The number of total genes detected by GeoChip analysis and overlapping genes between treatments were measured to understand the functional diversity and structure of the microbial communities. The number of total genes detected ranged from 27348 to 28491 and from 29311 to 33526 in AS and CT samples, respectively. An unpaired Student's *t*-test showed that these values were significantly different. The percentage of overlapping genes between samples ranged from 77.18% for AS (77.41, 75.25, and 78.88%) to 73.16% for CT (76.25, 65.70, and 77.52%) (Figure

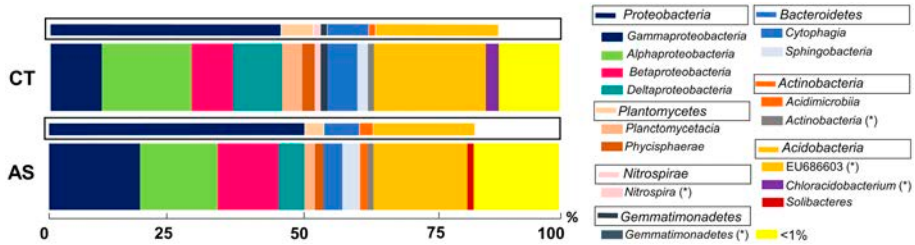


FIGURE 3 | Analysis of microbial communities present in field soil samples unamended (CT) and amended with composted almond shells (AS). Relative abundance (percentage) of different prokaryotic groups detected by 16S rRNA gene sequencing analysis of soil DNA. Analysis of microbial groups are marked at the class level (thick bars) and the phylum level (boxed thin bars). < 1%, sum of all detected groups with a relative abundance less than 1%. *Taxonomic characteristics of these groups are uncertain.

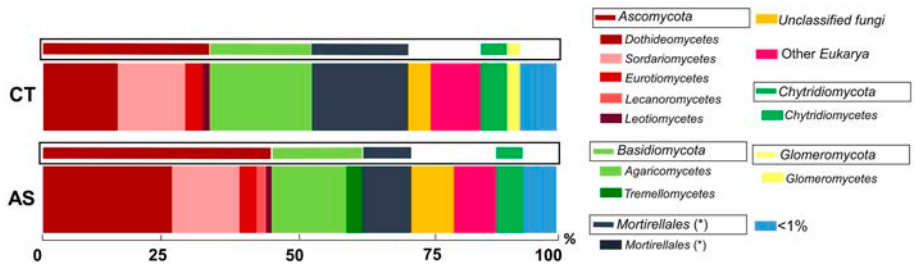


FIGURE 4 | Analysis of microbial communities present in field soil samples unamended (CT) and amended with composted almond shells (AS). Relative abundance (percentage) of different eukaryotic groups detected by ITS region sequence analysis of soil DNA. Analysis of microbial groups are marked at the class level (thick bars) and at phylum level (boxed thin bars). < 1%, sum of all detected groups with a relative abundance less than 1%. *Taxonomic characteristics of these groups are uncertain.

S3). This value fell to 65.43% when we compared overlapping genes between treatments (AS_{1-3} and CT_{1-3}). DCA (detrended correspondence analysis) and hierarchical clustering (with Bray-Curtis distance) were performed (Figure S3) using all of the detected genes, showing that functional structure of the microbial community was similar in the replicates but different among the soils (AS and CT).

To understand the effects of composted almond shells on the microbial communities and the acquired suppressive capacity, microbial functional genes categorized as participating in biogeochemical cycles and other important soil processes were examined (**Figure 5**). Gene functions related to the carbon cycle were the gene category most represented in all samples. C cycling probes were significantly more abundant than other categories in AS samples (36.65% in AS and 34.54% in CT), whereas genes related to organic contaminant degradation (12.42% in AS and 12.81% in CT), metal resistance (14.58% in AS and 16.32 in CT) and virulence (1.59% in AS and 1.61% in CT) were significantly more abundant in CT samples. There were no significant differences in N, P, and S cycle genes and other gene categories such as stress, fungi functions, soil benefit and soil borne pathogens (**Figure 5**).

Key genes for acetogenesis, C degradation, C fixation, methane metabolism, and other genes related to the C cycle were detected in the two types of soils (Figure S4A). The relative abundance of genes related to the C degradation category were the highest and exhibited significant differences between the AS samples and the CT samples. In this category, we found the presence of degradative genes of the most abundant C sources derived from plant and animal sources that could be present in soil ecosystems, such as starch, hemicellulose, cellulose, chitin, and lignin. There were few significant differences between samples in these categories of detected genes (Figure S4A).

Of the nitrogen cycle category, only the ammonification subcategory had a higher significant difference for amended soil (Figure S4B). In this subcategory, there are genes that function in the decomposition of organic matter and cycling of accumulated N.

Related to the sulfur cycle, the analyses performed exhibited a higher significant difference ($P < 0.1$) in only the sulphite reductase genes of AS samples compared to CT samples. These genes encode enzymes that catalyze the reduction of sulphite to sulfide, using iron as cofactor, and provide a source of S to microbiota. The CT samples exhibited a higher significant difference in sulfate reductase, a protein involved in sulfur reduction by anaerobic respiration (Figure S4C).

Statistical analyses showed no significant differences in the relative abundance of genes involved in the phosphorous cycle for these samples.

The analysis of genes in the category of environmental adaptability showed significant differences ($P < 0.1$) in the subcategories, as shown in Figures S4D–F. Genes involved in

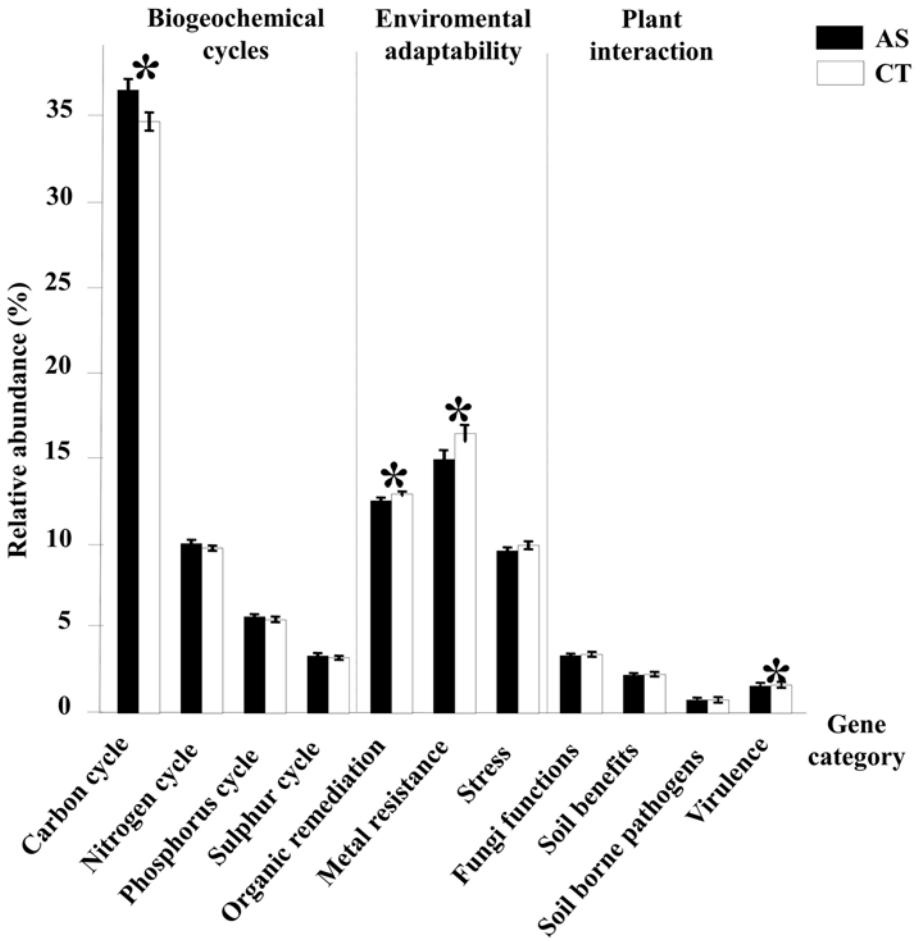


FIGURE 5 | GeoChip analysis of functional gene categories. Relative abundance of all detected genes from different gene categories analyzed in this study. *Indicates significant statistical differences ($p < 0.1$) between the two types of soil samples, amended soil (AS) and conventionally managed soil (CT). Standard error bars are shown.

the organic degradation of aromatics, such as chlorinated and pesticide-related compounds, had a higher significant relative abundance for amended soil than conventional managed soil. Similar results were obtained for genes related to osmotic and oxygen stress, from the stress category, and metal resistance to cobalt and lead, which had slightly higher significant relative abundance for AS samples than CT samples. On the other hand, unamended soils exhibited significantly higher values of relative abundance for genes related with stress induced by glucose limitation and metal resistance to cadmium and other metals.

The category of plant interaction covers a wide range of different functional genes involved in microbial interactions with plants, including genes related to fungal function, soil benefit, soil borne pathogens, and virulence. The analyses performed showed significant differences ($P < 0.1$) in some subcategories, as shown in Figures S4G–J. There were not any significant differences in the genes in the categories of soil benefit or fungi function. Nevertheless, CT samples exhibited a higher significant relative abundance of detected genes from the oomycetes subcategory (soil borne pathogen), which included different genes from this pathogenic group. Genes related to virulence processes such as iron oxidation or secretion had a higher significant relative abundance for amended soils; whereas unamended soils exhibited significantly higher values for genes involved in virulence actions such as iron uptake (aerobactin genes) and pilin formation.

Unique DNA Probes Detected in as Suppressive Soil Samples

Results of the GeoChip analysis and the Venn diagram representation allowed us to determine microbial specific gene functions detected exclusively in each treatment and the number of commonly detected probes (27364) (**Figure 6A**). We found 6674 unique detected probes in CT samples and 2766 unique detected probes in AS samples (approximately 10% of the total AS detected genes) from the gene categories analyzed. Approximately 34.49% of the unique hybridizations were related to the Carbon cycle category (**Figure 6B**), mainly to starch and chitin degradation (Table S1). The Organic remediation gene category exhibited 14.53% unique hybridizations of genes related

to the degradation of aromatic compounds. The Stress category had 13.38% unique hybridized probes and the Metal resistance category had 11.86% unique hybridized probes. The Nitrogen cycle category exhibited 8.57% unique hybridized probes, mostly in genes related with denitrification. The remaining gene categories had lower percentages: Sulfur cycle 5.60%, Fungi function 3.69%, Soil benefit 2.64% [approximately 44% of unique detected probes in this category correspond mainly with antimicrobial genes such as *cat* (catalase), *phzF* (phenazine), or *pcbC* (isopenicillin)], Phosphorus cycle 2.28%, Virulence 1.88%, and Soil borne pathogen 1.08% (**Figure 6B**). This analysis allowed us to relate different gene functions implicated in the metabolism of different soil compounds with bacterial or fungal classes present in the AS soil (Table S1).

DISCUSSION

The application of organic amendments to agricultural soils is a longstanding practice, and examples of organic-amendment-mediated suppression of soilborne diseases were reported as early as the late nineteenth century (Stone et al., 2004). Growers have observed that different types of organic materials suppress root rot for varying lengths of time. At present, nursery and greenhouse growers successfully use compost-amended potting mixes to suppress soilborne diseases, such as *Pythium* and *Phytophthora* root rots, in container systems (Hoitink et al., 1991). However, limited field studies have been conducted to determine the impact of soil amendments on microbial communities in actual organic and conventional production systems (Drinkwater et al., 1995; Gunapala and Scrow, 1998; Bulluck and Ristaino, 2001). In the case of avocado orchards, organic matter-mediated disease suppression against *Phytophthora cinnamomi* has been observed in avocado agricultural fields organically managed in Australia. Organic amendments (barley straw, sorghum residues, and native grass) were added to the soil under the trees as a mulch layer resulting in the suppression of *Phytophthora* root rot of avocado (Malajczuk, 1979, 1983). Additionally, our previous studies also demonstrated that different organic amendments can influence the composition and diversity of soil bacterial communities in avocado plants growing in microcosms after DGGE analysis, showing enhancement of specific populations such as *Burkholderia* and *Frateuria* (Bonilla et al., 2012a, 2015). Among different organic matter tested on avocado crops, composted almond shells (AS; commercial almond shells derived from the almond industry were piled and traditionally composted) exhibited enhancement of soil suppressiveness against *R. necatrix* (Bonilla et al., 2012a, 2015), the causal agent of avocado white root rot (Pliego et al., 2012). Even when soil suppressiveness against *R. necatrix* is improved after the addition of AS, only subtle changes in the bacterial community and composition and specific enzymatic activities have been reported using DGGE analysis (Bonilla et al., 2015). It must be considered that a wide range of factors can affect soil microorganism communities (van Veen et al., 1997). The soil samples used in our study came from the same orchard (same type of soil, environmental conditions, plant age, and cultivar, etc.), but were under different management, and this was assumed to be the only difference between the samples. The soil influenced by the amendment of AS showed some characteristics that differed from the conventional unamended soil. The almond shells are a high dry matter-containing substrate, composed of approximately 95% organic matter, with poor values of glucose, fructose, or sucrose. The characteristics and composition of AS makes this substrate an acceptable growing media for soilless culture (Valverde et al., 2013). Moreover, it must be taken into account that the avocado is a shallow rooted tree, with most of the feeder roots allocated in the top 15 cm, which needs good aeration. Roots are helped by the presence of a rich surface of organic mulch, as shown by the tendency of healthy feeder roots to grow into any decomposing litter layer (Chanderbali et al., 2013).

In this work, a metagenomic approach to the community composition of amended and unamended avocado soils have been performed for the first time. The use of metabarcoding and GeoChip techniques allowed a better knowledge on the community composition and their potential activities. In first place, an attempt to identify key factors involved in this enhanced suppressivity after the addition of organic amendments revealed the crucial role of the microbiota present in the organic amended soil. The microbiota evolved in the composted almond shells and was crucial for suppressiveness because the reduction of the bacterial population after a heat treatment in the organic amendment resulted in a more conducive phenotype (heat-treated soil samples harbor 10^5 cfu/g, most likely composed mainly by sporulated bacterial and fungal microorganisms). Moreover, total or partial suppressiveness was recovered when these heat-treated soil samples were complemented with a portion of soil influenced by AS, but it remained conducive when complemented with a portion of conventional soil (CT). This effect has been previously described for different suppressive soils, where sterilization by autoclaving, steam pasteurization, and irradiation rendered soils conducive to the pathogen studied (Malajczuk, 1983; Weller et al., 2002; Mendes et al., 2011). Suppressiveness experiments performed do not excluded the possibility that the disinfected avocado root used could harbor endophytic microorganisms, but our results significantly pointed out the role of the composted almond shells in the plant protection against *R. necatrix*. Thus, our results support the crucial role of microbes present in AS for turning the conducive CT soil into a more suppressive soil against *R. necatrix*.

To gain insights into the microbial diversity present in the soil samples, we used several different approaches. Phylogenetic marker analysis based on the sequencing of 16S rDNA and ITSs revealed a relatively similar array of prokaryotic and eukaryotic populations present in the AS and CT soil samples; however, a different response has been described in the literature for other types of organic matter from different sources, such as composted municipal waste (Zaccardelli et al., 2013). It is remarkable that in our model system, the group of fast-growing, easily cultivable *Proteobacteria* is the dominant group of prokaryotes in both soil samples. These data are similar to those previously observed for other soil and rhizosphere

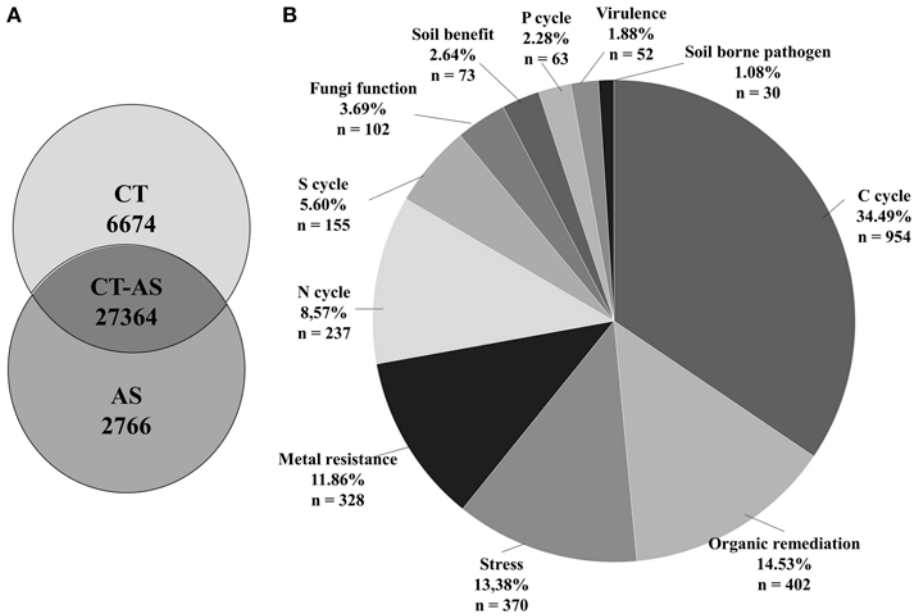


FIGURE 6 | GeoChip analysis of unique detected genes. (A) Number of core and unique detected genes (different gene ID) of amended soil (AS) and conventionally managed soil (CT). **(B)** Assigned functions of the unique genes detected in the AS sample.

samples with a high presence of organic matter (Lynch and Whipps, 1990; Paul and Clark, 1996; Hawkes et al., 2007; Mendes et al., 2011). Moreover, the representation of the other phyla different than *Proteobacteria* were quite similar among AS-amended and unamended soils, thus contradicting the idea that a highly specific community is stimulated by the addition of AS. Diversity analysis confirmed the previously obtained results (Bonilla et al., 2015), highlighting the enhancement of specific microbial populations in AS-amended samples, such as *Betaproteobacteria* (*Burkholderiales*) and the class of *Gammaproteobacteria*, which have been reported to protect plants from fungal infections in other suppressive soils (Mendes et al., 2011). It is important to note the clear enhancement in AS-amended soil of the order *Steroidobacter*, previously reported to play an essential role in the positive interactions with plants; for example, controlling seed germination, stem, and root elongation or stress protection in plants (Zarraonaindia et al., 2015).

In contrast, analysis of eukaryotic ITS revealed a different abundance distribution of microbes among the two types of soil samples. Fungal clones were the most common and dominant microbial eukaryotes in the soil. AS-amended soil samples had an increased relative abundance of *Ascomycota*. This fact is not surprising considering that *Ascomycetes* are the largest group on true fungi (Larena et al., 1999). Moreover, the dominance of *Ascomycota* has been observed during different composting processes (De Gannes et al., 2013; Neher et al., 2013), where most of them are saprophytic and live on dead organic material that they help decompose (Agrios, 1997; Viebahn et al., 2005). This behavior easily explains their higher abundance when composted almond shells are added to the soil as mulch. Within *Ascomycota*, the group that exhibited the most apparent and highest increase of abundance in AS-amended soil samples was the fungal class of *Dothideomycetes*. A high abundance of *Dothideomycetes* in soils with at high hydrocarbon concentrations has been previously reported (Ferrari et al., 2011), suggesting its preference for those habitats with a high concentration of organic matter where it participates in biomass conversion (Shrestha et al., 2011). Moreover, the large increase of the phylum *Pleosporales* (*Dothideomycetes*) is also not surprising because this group is very well-known to contain species that chlorinate lignin as a first step of biomass conversion during plant litter degradation (Ortíz-Bermúdez et al., 2007). Interestingly, it has been shown that several genera of *Dothideomycetes* exhibit an increased presence in suppressive soils because they harbor endohyphal bacteria from groups that are capable of hydrocarbon biodegradation, such as the *Xanthomonadales*, *Pseudomonadales*, *Burkholderiales*, and *Sphingomonadales* (Hoffman and Arnold, 2010). *Dothideomycetes* have also been shown to increase slightly in AS-amended soils. However, the group that shows an apparent decrease in AS-amended soils is *Mortierellales*. This group has a complex phylogeny (Wagner et al., 2013) and is considered to be ubiquitous in the bulk and rhizospheric soil, implying that it could play a role in maintenance of the micro-ecological balance (Miao et al., in press). Interestingly, the group of *Glomeromycota*, which contains different groups of symbiotic fungi previously detected in avocado (Hass and Menge, 1990; González-Cortés et al., 2012), it is clearly detected in unamended soils, but decreased in the amended ones (below 1%). A possible explanation could be that in the AS amended soils, take place a strong competition with other decomposing fungi, such as the *Dothideomycetes*, more adapted to an environment with high amount of decomposing organic matter. Finally, it should be noted that members of *Xylariaceae*, to which *R. necatrix* belongs

(Pliego et al., 2012), are less abundant in AS-amended soils, thus revealing a negative effect on this fungal group. These results indicate that the soil fungal community was affected by the soil amendment with AS.

Phylogenetic markers such as the prokaryotic 16S and eukaryotic ITS region do not carry explicit functional information. For this, the use of GeoChip-based analysis allowed for the analysis of microbial functional genes encoding key enzymes involved in major biogeochemical processes that facilitate linking microbial community structure to potential ecological functions (Torsvik and Ovreas, 2002). Using this technique, we screened potential functional gene diversity among unamended and AS-amended soil samples.

Probe signals and DCA analysis indicated that the microbial community functional structures differed between CT and AS soil samples. The sample sites are very close together, so the differences observed in the microbial communities are thought to be the result of amendment with organic matter.

Generally, similar abundance patterns of functional genes involved in nutrient cycling processes such a nitrogen, phosphorous or sulfur cycling, were found in both types of samples. However, AS-amended samples had higher signal intensities for C degradation (carbon cycle) genes than CT, with some differences being statistically significant. Substrates for this group of genes ranged from labile C to more recalcitrant C (e.g., starch, hemicelluloses, cellulose, chitin, and lignin). These results suggest that AS-amended microbial have a greater capacity for C degradation than CT communities. This suggests, as expected, an important role of carbon cycling in response to the addition of organic matter to the soil. However, no differences in gene abundance for N, P, or S cycling was observed. This can be explained because almond shells are a lignin-rich waste resulting from the almond industry, mostly composed of approximately 27% lignin and 73% holocellulose (Caballero et al., 1996), and those cycles were not compromised. However, statistical differences in the abundance of genes related to organic remediation and metal resistance were observed in AS-amended soil displaying lower levels than CT. This observation may be due to a decrease in the available compounds due to the high sorption ability of the composted almond shells and derivate compounds from its degradation, which have been previously reported to be able to remove such substances from the soil (Pehlivan et al., 2009).

Interestingly, both soil samples shared a core of probes corresponding to approximately 90% of the assayed sequences (27364 probes). However, approximately 10% of the total probes analyzed were unique for AS-amended samples (2766 probes). When the sequence of these probes were analyzed, they resulted in a very similar distribution to that previously shown for the whole GeoChip analysis, with above 34.5% corresponding to C cycling, followed by probes related to organic remediation (14.5%), stress (13.4%), metal resistance (11.9%), or the N cycle (8.6%). These results support the following previously described results: systems associated with organic matter-mediated general suppression; suppression typically occurs as a result of the activation of the indigenous microbial community (Lockwood, 1990); and suppressive activities can be generated by one to few populations of organisms (Gerlagh, 1968; Cook and Baker, 1983; Hoitink and Boehm, 1999; Weller et al., 2002). Postma et al. (2000) found that qualitative rather than quantitative shifts in the bacterial community correlate with disease suppressiveness, and several studies indicated that mechanisms within the microbial activity of the soil are responsible for the suppression of pathogens (Rovira and Wildermuth, 1981; Nitta, 1991; Workneh and van Bruggen, 1994; van Os and van Ginkel, 2001).

Among the specific taxa stimulated, *Pseudomonadaceae*, *Burkholderiaceae*, *Xanthomonadales*, and *Actinobacteria*, harbor genera and species with activity against plant pathogenic fungi (Postma et al., 2010). Additionally, it is important to note that *Pseudomonas, Rhizobium, Bacillus, Variovorax, Phyllobacterium,* and *Azospirillum*, are considered the most efficient plant growth-promoting bacteria (Bertrand et al., 2001).

Sequencing of specific probes present in AS-amended soils revealed the presence in such soil samples of genes for bacterial and fungal catalases, phenazine biosynthetic genes (from *Proteobacteria*) or the presence of potential antibiotics produced by *Actinobacteria* (data not shown). Nearly all of these probes corresponded to the GeoChip category "soil benefit," where the antimicrobials from different groups were analyzed. To the best of our knowledge, no probes from *Bacilli* were used, so the role of antimicrobials such as iturin or fengicins, produced by *Bacillus* spp., cannot be discussed based on our results.

It is important to note that the genus *Pseudomonas* (class *Gammaproteobacteria*) and *Bacillus* (class *Bacilli*) are two of the most prominent bacteria that can be isolated from avocado soil and rhizosphere displaying antifungal activity and plant protection against soil-borne pathogens (Cazorla et al., 2006, 2007; González-Sánchez et al., 2010). Our results reinforce the importance of such microorganisms in the soil and root ecology of the avocado crop. These groups of microorganisms can produce metabolites, such as siderophores and antibiotics, with specific suppressive activity against soilborne pathogens. Antagonistic pseudomonads, including *Pseudomonas chlororaphis*, play a role in white root rot suppressiveness (Cazorla et al., 2006; Calderón et al., 2014). However, other types of rhizobacterial taxa may differ in prevalence between suppressive and conducive soils, suggesting that the microbial basis of white root rot could be far more complex than solely a *Pseudomonas* property; it has also been observed for other pathosystems such as *Thielaviospsis basicola*-mediated black root rot of tobacco (Almario et al., 2014).

In conclusion, and taking together the results obtained in this work and in previous works related, a theoretical model about the role of the microorganisms in enhancing suppressiveness after amendment with composted almond shells can be proposed (Figure S5). Soil amendments with composted almond shells resulted in an extra input of organic matter rich in lignin that could be initially degraded by fungal members of the community (such as *Dothideomycetes*) and Actinobacterias. Lignin degradation from composting almond shells would produce a progressive release to the soil of more simple compounds. Those compounds, together with others also present in the almond shells, could lead to an increase in carbon

sources available, such as cellulose, hemicellulose, and aromatic compounds. At this point, some *Proteobacteria* already present in the soil (such as *Gammaproteobacteria* and *Betaproteobacteria*) could take advantage metabolizing that available organic matter, thus slightly enhancing their population. These groups of microorganisms could harbor, among other, genes involved in antifungal enzymatic activities and production of antimicrobial compounds that could have an effect on the interaction with other microbes. The resulting modified microbiota after addition of composted almond shells could be more active against some groups of phytopathogenic fungi (as *Xilariales,* where *R. necatrix* is included) finally showing a phenotype of induced suppressiveness effect.

AUTHOR CONTRIBUTIONS

FC and AD designed and corrected the manuscript. NB contributed to data management. CV performed date management and wrote the manuscript.

ACKNOWLEDGMENTS

We want to thank José M. Hermoso, Jorge González, and Emilio Guirado for assistance in suppressive assay design and management. We would like to thank Irene Linares and Claudia E. Calderón for their assistance during various parts of the projects. We are especially grateful to José M. Farré and Juan A. Torés for their helpful ideas and discussion, which were essential for this project. This work was supported by Plan Nacional I+D+I from Ministerio de Economia (MINECO) (AGL11-30354-C02-01 and AGL14-52518-C2-1-R), co-financed by FEDER funds (EU). CV was supported by a PhD fellowship from the FPI program of MINECO, and NB was supported by a PhD fellowship from the FPU program of MICINN.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00004

REFERENCES

Agrios, G. N. (ed.). (1997). *Plant Pathology*. San Diego, CA: Academic Press.

Almario, J., Muller, D., Défago, G., and Moënne-Loccoz, Y. (2014). Rhizosphere ecology and phytoprotection in soils naturally suppressive to Thielaviopsis black root rot of tobacco. *Environ. Microbiol.* 16, 1949–1960. doi: 10.1111/1462-2920.12459

Bai, S., Li, J., He, Z., van Nostrand, J. D., Tian, Y., Lin, G., et al. (2013). GeoChip-based analysis of the functional gene diversity and metabolic potential of soil microbial communities of mangroves. *Appl. Microbiol. Biotechnol.* 97, 7035–7048. doi: 10.1007/s00253-012-4496-z

Bailey, K. L., and Lazarovits, G. (2003). Suppressing soil-borne diseases with residue management and organic amendments. *Soil Tillage Res.* 72, 169–180. doi: 10.1016/S0167-1987(03)00086-2

Bender, G. S., Casale, W. L., and Rahimian, M. (1992). "Use of worm-composted sludge as a soil amendment for avocados in Phytophthora-infested soil," in *Proceeding of Second World Avocado Congress* (Orange, CA), 143.

Bertrand, H., Nalin, R., Balli, E., and Cleyet-Maret, J. C. (2001). Isolation and indentification of the most efficient plant growth-promoting bacteria associated to canole *(Brassica napus). Biol. Fertil. Soils* 33, 152–156. doi: 10.1007/s003740000305

Bonanomi, G., D'Ascoli, R., Scotti, R., Gaglione, S. A., Caceres, M. G., Sultana, S., et al. (2014). Soil quality recovery and crop yield enhancement by combined application of compost and wood to vegetables grown under plastic tunnels. *Agric. Ecosyst. Environ.* 192, 1–7. doi: 10.1016/j.agee.2014.03.029

Bonilla, N., Cazorla, F. M., Martínez-Alonso, M., Hermoso, J. M., González-Fernández, J. J., Gaju, N., et al. (2012a). Organic amendments and land management affect bacterial community composition, diversity and biomass in avocado crop soils. *Plant Soil* 357, 215–226. doi: 10.1007/s11104-012-1155-1

Bonilla, N., Gutiérrez-Barranquero, J., Vicente, A., and Cazorla, F. (2012b). Enhancing soil quality and plant health through suppressive organic amendments. *Diversity* 4, 475–491. doi: 10.3390/d4040475

Bonilla, N., Vida, C., Martínez-Alonso, M., Landa, B. B., Gaju, N., Cazorla, F. M., et al. (2015). Organic amendments to avocado crops induce suppressiveness and influence the composition and activity of soil microbial communities. *Appl. Environ. Microbiol.* 81, 3405–3418. doi: 10.1128/AEM.03787-14

Bulluck, L. R. III, and Ristaino, J. B. (2001). Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. *Phytopathology* 92, 181–189. doi: 10.1094/PHYTO.2002.92.2.181

Caballero, J. A., Font, R., and Marcilla, A. (1996). Comparative study of the pyrolysis of almond shells and their fractions, holocellulose and lignin. Product yields and kinetics. *Thermochim. Acta* 276, 57–77. doi: 10.1016/0040-6031(95)02794-7

Calderón, C. E., de Vicente, A., and Cazorla, F. M. (2014). Role of 2-hexyl, 5-propyl resorcinol production by *Pseudomonas chlororaphis* PCL1606 in the multitrophic interactions in the avocado rhizosphere during the biocontrol process. *FEMS Microbiol. Ecol.* 89, 20–31. doi: 10.1111/1574-6941.12319

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Cazorla, F. M., Duckett, S. B., Bergström, E. T., Noreen, S., Odijk, R., Lugtenberg, B. J. J., et al. (2006). Biocontrol of avocado dematophora root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-hexyl 5-propyl resorcinol. *Mol. Plant Microbe Interact.* 19, 418–428. doi: 10.1094/MPMI-19-0418

Cazorla, F. M., Romero, D., Pérez-García, A., Lugtenberg, B. J. J., de Vicente, A., and Bloemberg. (2007). Isolation and characterization of antagonistic *Bacillus subtilis* strains from the avocado rhizoplane displaying biocontrol activity. *J. Appl. Microbiol.* 103, 1950–1959. doi: 10.1111/j.1365-2672.2007.03433.x

Chanderbali, A. S., Soltis, D. E., Soltis, P. S., and Wolstenholme, B. N. (2013). "Taxonomy and Botany," in *The Avocado: Botany, Production and Uses, 2nd Edn*, eds B. A. Schaffer, B. N. Wolstenholme, and A. W. Whiley (Oxfordshire: CABI), 31–50. doi: 10.1079/9781845937010.0031

Cong, J., Yang, Y., Liu, X., Lu, H., Liu, X., Zhou, J., et al. (2015). Analyses of soil microbial community compositions and functional genes reveal potential consequences of natural forest succession. *Sci. Rep.* 5:10007. doi: 10.1038/srep10007

Cook, R. J., and Baker, F. (1983). *The Nature and Practice of Biological Control of Plant Pathogens*. Saint Paul, MN: The American Phytopathological Society.

Cretoiu, M. S., Korthals, G. W., Visser, J. H. M., and van Elsas, J. D. (2013). Chitin amendment increases soil suppressiveness toward plant pathogens and modulates the actinobacterial and oxalobacteraceal communities in an experimental agricultural field. *Appl. Environ. Microbiol.* 79, 5291–5301. doi: 10.1128/AEM.01361-13

De Gannes, V. D., Endoxic, G., and Hickey, W. J. (2013). Insight into fungal communities in compost revealed by 454-pyrosequencing: implications for human health and safety. *Front. Microbiol.* 4:164. doi: 10.3389/fmicb.2013.00164

Deng, Y., and Zhou, J. (2013). "Molecular ecological network of microbial communities," in *Encyclopedia of Metagenomics,* ed K. Nelson (Berlin; Heidelberg: Springer-Verlag), 1–8. doi: 10.1007/978-1-4614-6418-1_758-2

Downer, A. J., Menge, J. A., and Pond, E. (2001). Association of cellulolytic enzyme activities in Eucalyptus mulches with biological control of *Phytophthora cinnammomi*. *Phytopathology* 91, 847–855. doi: 10.1094/PHYTO.2001.91.9.847

Drinkwater, L. E., Letourneau, D. K., Workneh, F., van Bruggen, A. H. C., and Shennan, C. (1995). Fundamental differences between conventional and organic tomato agroecosystems in California. *Ecol. Appl.* 5, 1098–1112. doi: 10.2307/2269357

Ferrari, B. C., Zhang, C., and van Dorst, J. (2011). Recovering greater fungal diversity from pristine and diesel fuel contaminated sub-Antartic soil through cultivation using both a high and a low nutrient media approach. *Front. Microbiol.* 2:217. doi: 10.3389/fmicb.2011.00217

Freeman, S., Sztejnberg, A., and Chet, I. (1986). Evaluation of *Trichoderma* as a biocontrol agent for *Rosellinia necatrix*. *Plant Soil* 94, 163–170. doi: 10.1007/BF02374340

Garbeva, P., Veen, J. A., and van Elsas, J. D. (2004). Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. *Ann. Rev. Phytopathol.* 42, 243–270. doi: 10.1146/annurev.phyto.42.012604.135455

Gerlagh, M. (1968). Introduction of *Ophiobolus graminis* into new polders and its decline. *Neth. J. Plant Pathol.* 74, 1–97. doi: 10.1007/BF02019999

González-Cortés, J. C., Vega-Fraga, M., Varela-Fregoso, L., Martínez-Trujillo, M., Carreón-Abud, Y., and Gavito, M. E. (2012). Arbuscular mycorrhizal fungal (AMF) communities and land use change: the conversion of temperate forest to avocado plantations and maize fields in central Mexico. *Fungal Ecol.* 5, 16–23. doi: 10.1016/j.funeco.2011.09.002

González-Sánchez, M. A., de Vicente, A., Pérez-García, A., Pérez-Jiménez, R., Romero, D., and Cazorla, F. M. (2013). Evalation of the effectiveness of biocontrol bacteria against avocado white root rot occurring under commercial greenhouse plant production conditions. *Biol. Cont.* 67, 94–100. doi: 10.1016/j.biocontrol.2013.08.009

González-Sánchez, M. A., Pérez-Jiménez, R. M., Pliego, C., Ramos, C., de Vicente, A., and Cazorla, F. M. (2010). Biocontrol bacteria selected by a direct plant protection strategy against avocado white root rot show antagonism as a prevalent trait. *J. Appl. Microbiol.* 109, 65–78. doi: 10.1111/j.1365-2672.2009.04628.x

Gunapala, N., and Scrow, K. M. (1998). Dynamics of soil microbial biomass and activity in conventional and organic farming systems. *Soil Biol. Biochem.* 30, 805–816. doi: 10.1016/S0038-0717(97)00162-4

Hadar, Y., and Papadopoulou, K. K. (2012). Suppressive composts: microbial ecology links between abiotic environments and healthy plants. *Annu. Rev. Phytopathol.* 50, 133–153. doi: 10.1146/annurev-phyto-081211-172914

Hass, J. H., and Menge, J. A. (1990). VA-mycorrhizal fungi and soil characteristics in avocado (Persea Americana Mill.) orchard soils. *Plant Soil* 127, 207–212. doi: 10.1007/BF00014427

Hawkes, C. V., DeAngelis, K. M., and Firestone, M. K. (2007). "Root interactions with soil microbial communities and processes," in *The Rhizosphere,* Vol. 1, eds Z. Cardon, and J. Whitbeck (New York, NY: Elsevier), 1–29.

Hazen, T. C., Dubinsky, E. A., DeSantis, T. Z., Andersen, G. L., Piceno, Y. M., Singh, N., et al. (2010). Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* 330, 204–208. doi: 10.1126/science.1195979

He, Z., Deng, Y., and Zhou, J. (2012). Development of functional gene microarrays for microbial community analysis. *Curr. Opin. Biotechnol.* 23, 49–55. doi: 10.1016/j.copbio.2011.11.001

Hermoso, J. M., Torres, M. D., and Farré, J. M. (2011). "Effects of organic materials on adult Hass trees," in *Proceedings of the Seventh World Avocado Congress* (Cairns, QLD).

Hirsch, P. R., Mauchline, T. H., and Clark, I. M. (2013). "Culture-independent molecular approaches to microbial ecology in soil and the rhizosphere," in *Molecular Microbial Ecology of the Rhizosphere,* Vol. 5, ed F. J. de Bruinjn (Hoboken, NJ: John Wiley & Sons, Inc). 45–55. doi: 10.1002/9781118297674.ch5

Hoffman, M. T., and Arnold, A. E. (2010). Diverse bacteria inhabit living hyphae of phylogenetically diverse fungal endophytes. *Appl. Environ. Microbiol.* 76, 4063–4075. doi: 10.1128/AEM.02928-09

Hoitink, H., and Boehm, M. (1999). Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon. *Annu. Rev. Phytopathol.* 37, 427–446. doi: 10.1146/annurev.phyto.37.1.427

Hoitink, H., Stone, A. G., and Han, D. Y. (1991). Suppression of plant diseases by composts. *HortScience* 32, 184–187.

Janvier, C., Villeneuve, F., Alabouvette, C., Edel-Hermann, V., Mateille, T., and Steinberg, C. (2007). Soil health through soil disease suppression: which strategy from descriptors to indicators? *Soil Biol. Biochem.* 39, 1–23. doi: 10.1016/j.soilbio.2006.07.001

Koyama, A., Wallenstein, M., Simpson, R. T., and Moore, J. C. (2014). Soil bacterial community composition altered by increased nutrient availability in Arctic tundra soils. *Front. Microbiol.* 5:516. doi: 10.3389/fmicb.2014.00516

Larena, I., Salazar, O., González, V., Julián, M. C., and Rubio, V. (1999). Design of a primer for ribosomal DNA internal transcriber spacer with enhanced specificity for *Ascomycetes*. *J. Biotech.* 75, 187–194. doi: 10.1016/S0168-1656(99)00154-6

Lazarovits, G., Tenuta, M., and Conn, K. (2001). Organic amendments as a disease control strategy for soilborne diseases of high-value agricultural crops. *Australas. Plant. Pathol.* 30, 111–117. doi: 10.1071/AP01009

Liang, Y., He, Z., Wu, L., Deng, Y., Li, G., and Zhou, J. (2010). Development of a common oligonucleotide reference standard for microarray data normalization and comparison across different microbial communities. *Appl. Environ. Microbiol.* 76, 1088–1094. doi: 10.1128/AEM.02749-09

Lockwood, J. L. (1990). "Relation of energy stress to behavior of soil-borne plant pathogens and to disease development," in *Biological Control of Soil-borne Plant Pathogens*, eds D. Hornby and R. J. Cook (Wallinford, CT: CAB International), 197–214.

López, R., Burgos, P., Hermoso, J. M., Hormaza, J. I., and González-Fernández, J. J. (2014). Long term changes in soil properties and enzyme activities after almond shell mulching in avocado organic production. *Soil Tillage Res.* 143, 155–163. doi: 10.1016/j.still.2014.06.004

López-Herrera, C. J., Pérez-Jiménez, R. M., Zea-Bonilla, T., Basallote-Ureba, M. J., and Melero-Vara, J. M. (1998). Soil solarization in established avocado trees for control of *Dematophora necatrix*. *Plant Dis.* 82, 1088–1092. doi: 10.1094/PDIS.1998.82.10.1088

López-Herrera, C. J., and Zea-Bonilla, T. (2007). Effects of benomyl, carbendazim, fluazinam and thiophanate methyl on white root rot of avocado. *Crop Prot.* 26, 1186–1192. doi: 10.1016/j.cropro.2006.10.015

Lynch, J. M., and Whipps, J. M. (1990). Substrate flow in the rhizosphere. *Plant Soil* 129, 1–10. doi: 10.1007/BF00011685

Malajczuk, N. (1979). "Biological suppression of *Phytophthora cinnamomi* in eucalypts and avocados in Australia," in *Soil-borne Plant Pathogens*, eds B. Schippers and W. Gams (London: Academic Press), 635–652.

Malajczuk, N. (1983). "Microbial antagonism to Phytophthora," in *Phytophthora, Its Biology, Taxonomy, Ecology and Pathology,* eds D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao (Saint Paul, MN: American Phytopathological Society), 197–218.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Shneider, J. H. M., et al. (2011). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* 332, 1097–1100. doi: 10.1126/science.1203980

Miao, C. P., Mi, Q. L., Qiao, X. G., Zheng, Y. K., Chen, Y. W., Xu, L., et al. (in press). Rhizospheric fungi of *Panax notoginseng*: diversity and antagonism to host phytopathogens. *J. Ginseng Res.* doi: 10.1016/j.jgr.2015.06.004

Moore-Gordon, C., Cowan, A. K., and Wolstenholme, B. N. (1997). "Mulching of avocado orchards to increase Hass yield and fruit size and boost financial rewards a three season summary of research findings," in *South African Avocado Growers' Association Yearbook,* Vol. 20 (Tzanee: SAAGA) 46–49.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H., and Wawer, C. (2004). "Denaturing gradient gel electrophoresis (DGGE) in microbial ecology," in *Molecular Microbial Ecology,* Vol. 1, eds G. A. Kowalchuk, F. J. Bruinjn, I. M. Heas, A. D. L. Akkermans, and J. D. van Elsas (Dordrecht: Kluwer Academic Publications), 743–770.

Neher, D. A., Weicht, T. R., Bates, S. T., Left, J. W., and Fierer, N. (2013). Changes in bacterial and fungal communities across compost recipes, preparation method, and composting times. *PLoS ONE* 8:e79512. doi: 10.1371/journal.pone.0079512

Nitta, T. (1991). Diversity of root fungal floras: its implications for soil-borne diseases and crop growth. *Jpn. Agric. Res.* 25, 6–11.

Ortíz-Bermúdez, P., Hirth, K. C., Srebotnik, E., and Hammel, K. E. (2007). Chlorination of lignin by ubiquitous fungi has a likely role in global

organichloride production. *Proc. Natl. Acad. Sci. U.S.A.* 104, 3895–3900. doi: 10.1073/pnas.0610074104

Pane, C., Piccolo, A., Spaccini, R., Celano, G., Villecco, D., and Zaccardelli, M. (2013). Agricultural waste-based composts exhibiting suppressivity to diseases caused by the phytopathogenic soil-borne fungi *Rhizoctonia solani* and *Sclerotinia minor*. *Appl. Soil Ecol.* 65, 43–51. doi: 10.1016/j.apsoil.2013.01.002

Paul, E. A., and Clark, F. E. (1996). *Soil Microbiology and Biochemistry, 2nd Edn.* San Diego, CA: Academic Press, Inc.

Pehlivan, E., Altun, T., Cetin, S., and Bhanger, M. I. (2009). Lead sorption by waste biomass of hazelnut and almond shell. *J. Hazard. Mater.* 167, 1203–2128. doi: 10.1016/j.jhazmat.2009.01.126

Peiffer, J. A., Spor, A., Koren, O., Jin, Z., Green, S., and Dangl, J. L. (2013). Diversity and heritability of the maize rhizosphere microbiome under field conditions. *Proc. Natl. Acad. Sci. U.S.A.* 110, 6548–6553. doi: 10.1073/pnas.1302837110

Pliego, C., Kanematsu, S., Ruano-Rosa, D., de Vicente, A., López-Herrera, C., Cazorla, F. M., et al. (2009). GFP sheds light on the infection process of avocado roots by *Rosellinia necatrix*. *Fungal Genet. Biol.* 46, 137–145. doi: 10.1016/j.fgb.2008.11.009

Pliego, C., López-herrera, C., Ramos, C., and Cazorla, F. M. (2012). Developing tools to unravel the biological secrets of *Rosellinia necatrix*, an emergent threat to woody crops. *Mol. Plant Pathol.* 13, 226–239. doi: 10.1111/j.1364-3703.2011.00753.x

Postma, J., Sheper, R. W. A., and Schilder, M. T. (2010). Effect of successive cauliflower plantings and *Rhizoctonia solani* AG 2-1 inoculations on disease suppressiveness of a suppressive and conducive soil. *Soil Biol. Biochem.* 42, 804–812. doi: 10.1016/j.soilbio.2010.01.017

Postma, J., Willemsen-de Klein, M. J., and van Elsas, J. D. (2000). Effect of the indigenous microflora on the development of root and crown rot caused by *Pythium aphanidermatum* in cucumber grown on rockwool. *Phytopathology* 90, 125–133. doi: 10.1094/PHYTO.2000.90.2.125

Rovira, A. D., and Wildermuth, G. B. (1981). "The nature and mechanisms of suppression," in *Biology and Control of Take All,* eds M. J. C. Asher and P. Shipton (London: Academic), 385–415.

Ruano-Rosa, D., and López-Herrera, C. J. (2009). Evaluation of *Trichoderma* spp. As biocontrol agents against avocado white root rot. *Biol. Cont.* 51, 66–71. doi: 10.1016/j.biocontrol.2009.05.005

Ruano-Rosa, D., Martín-Pérez, R., Bonilla, N., Cazorla, F. M., de Vicente, A., and López-Herrera, C. J. (2014). Biological control of avocado white root rot with combined applications of *Trichoderma* spp. and rhizobacteria. *Europ. Plant Pathol.* 138, 751–762. doi: 10.1007/s10658-013-0347-8

Shrestha, K., Shrestha, P., Walsh, K. B., Harrower, K. M., and Midmore, D. J. (2011). Microbial enhancement of compost extracts based on cattle rumen content. compost-characterisation of a system. *Bioresour. Technol.* 102, 8027–8034. doi: 10.1016/j.biortech.2011.06.076

Sokal, R. R., and Rohlf, F. J. (1986). *Introducción a la bioestadística*. Barcelona: Reverté.

Stone, A. G., Scheuerell, S. J., and Darby, H. M. (2004). "Suppression of soilborne diseases in field agricultural systems: organic matter management, cover cropping, and other cultural practices," in *Soil Organic Matter in Sustainable Agriculture,* eds F. Magdoff and R. R. Well (Boca Ratón, FL: CRC Press), 131–177. doi: 10.1201/9780203496374.ch5

Termorshuizen, A. J., van Rijn, E., van der Gaag, D. J., Alabouvette, C., Chen, Y., Lagerföf, J., et al. (2006). Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. *Soil Biol. Biochem.* 38, 2461–2477. doi: 10.1016/j.soilbio.2006.03.002

Torsvik, V., and Ovreas, L. (2002). Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5, 240–224. doi: 10.1016/S1369-5274(02)00324-7

Tu, Q., Yu, H., He, Z., Deng, Y., Wu, L., van Nostrand, J. D., et al. (2014). GeoChip 4: a functional gene-array-based high-throughput environmental technology for microbial community analysis. *Mol. Ecol. Resour.* 14, 914–928. doi: 10.1111/1755-0998.12239

Valverde, M., Madrid, R., García, A. L., del Amor, F. M., and Rincón, L. (2013). Use of almond shell and almond hull as substrate for sweet pepper cultivation. Effect on fruit yield and mineral content. *Span. J. Agric. Res.* 11, 164–172. doi: 10.5424/sjar/2013111-3566

van Elsas, J. D., Costa, R., Jansson, J., Sjöling, S., Bailey, M., Nalin, R., et al. (2008). The metagenome of disease suppressive soils—experiences from the METACONTROL project. *Trends Biotecnol.* 26, 591–601. doi: 10.1016/j.tibtech.2008.07.004

van Elsas, J. D., Jansson, J. K., and Trevors, T. (eds.). (2007). *Modern Soil Microbiology II*. Boca Raton, FL: CRC Press, Taylor & Francis Group, LLC.

van Elsas, J. D., and Postma, J. (2007). "Suppression of soil-borne phytopathogens by compost," in *Compost Science and Technology,* Vol. 8, eds L. F. Diaz, M. de Bertoldi, W. Bidlingmaier, and E. Stentiford (Amsterdam: Elsevier), 201–214.

van Os, G. J., and van Ginkel, J. H. (2001). Suppression of Pythium root rot in bulbous iris in relation to biomass and activity of the soil microflora. *Soil Biol. Biochem*. 32, 1447–1454. doi: 10.1016/S0038-0717(01)00053-0

van Veen, J. A., van Oberbeek, L. S., and van Elsas, J. D. (1997). Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. Rev.* 61, 121–135.

Viebahn, M. C., Veeman, C., Wernars, K., van Loon, L. C., Smith, E., and Bakker, P. A. H. M. (2005). Assessment of differences in *ascomycete* communities in the rhizosphere of field-grown wheat and potato. *FEMS Microbiol. Ecol.* 53, 245–253. doi: 10.1016/j.femsec.2004.12.014

Wagner, L., Stielow, B., Hoffmann, K., Petkovits, T., Papp, T., Vágvölgyi, C., et al. (2013). A comprehensive molecular phylogeny of the *Mortierellales* (*Mortierellomycotina*) based on nuclear ribosomal DNA. *Persoonia* 30, 77–93. doi: 10.3767/003158513X666268

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu. Rev. Phytopathol.* 40, 309–348. doi: 10.1146/annurev.phyto.40.030402.110010

Wolstenholme, B. N., Moore-Gordon, C. S., and Cowan, A. K. (1997). "Orchard mulching effects on avocado fruiting," in *Proceedings from the Australian Avocaco Growers' Association Conference'97: Searching for Quality*, ed I. G. Cutting (Rotorna), 119–130.

Workneh, F., and van Bruggen, A. H. C. (1994). Microbial densitiy, composition, and diversity in organically and conventionally managed rhizosphere soil in relation to suppression of corky root of tomatoes. *Appl. Soil Ecol.* 1, 219–230. doi: 10.1016/0929-1393(94)90013-2

Xie, J., He, Z., Liu, X., Liu, X., Van Nostrand, J. D., Deng, Y., et al. (2011). GeoChip-based analysis of the functional gene diversity and metabolic potential of microbial communities in acid mine drainage. *Appl. Environ. Microbiol.* 77, 991–999. doi: 10.1128/AEM.01798-10

Xue, K., Wu, L., Deng, Y., He, Z., van Nostrand, J., Robertson, P. G., et al. (2013). Functional gene differences in soil microbial communities from conventional, low-input, and organic farmlands. *Appl. Environ. Microbiol.* 79, 1284–1292. doi: 10.1128/AEM.03393-12

Zaccardelli, M., de Nicola, F., Villecco, D., and Scotti, R. (2013). The development and suppressive activity of soil microbial communities under compost amendment. *J. Soil Sci. Plant Nutr.* 13, 730–742. doi: 10.4067/s0718-95162013005000058

Zarraonaindia, I., Owens, S. M., Weisenhorn, P., West, K., Hampton-Marcell, J., Lax, S., et al. (2015). The soil microbiome influences grapevine-associated microbiota. *MBio* 6:e02527-14. doi: 10.1128/mBio.02527-14

Zhang, Y., Lu, Z., Liu, S., Yang, Y., He, Z., Ren, Z., et al. (2013). GeoChip-based analysis of microbial communities in alpine meadow soils in the Qinghai-Tibetan plateau. *BMC Microbiol.* 13:72. doi: 10.1186/1471-2180-13-72

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

jga-genome/jga99917/jga5104d17z | xppws | S=3 | 2/13/17 | 10:48 | ArtID: | DOI:10.1128/genomeA.00130-17 | CE: MT

Editor: | Section: **Prokaryotes** | Designation:

PROKARYOTES

AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements™

Draft Genome Sequence of the Rhizobacterium *Pseudomonas chlororaphis* PCL1601, Displaying Biocontrol against Soilborne Phytopathogens

AQ: au **Carmen Vida, Antonio de Vicente, Francisco M. Cazorla**

AQ: aff Instituto de Hortofruticultura Subtropical y Mediterránea La Mayora, Universidad de Málaga, Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC); Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain

ABSTRACT In this study, we present the draft genome sequence of the bacterial strain *Pseudomonas chlororaphis* PCL1601. This bacterium was isolated from the rhizosphere of healthy avocado trees and displayed antagonistic and biological control activities against different soilborne phytopathogenic fungi and oomycete.

Received 6 February 2017 **Accepted** 7 February 2017 **Published** XXX

Citation Vida C, de Vicente A, Cazorla FM. 2017. Draft genome sequence of the rhizobacterium *Pseudomonas chlororaphis* PCL1601, displaying biocontrol against soilborne phytopathogens. Genome Announc 0(999):e00130-17. https://doi.org/10.1128/genomeA.00130-17.

Address correspondence to Francisco M. Cazorla, cazorla@uma.es.

Pseudomonas chlororaphis PCL1601 is a Gram-negative aerobic bacterium isolated from the rhizosphere of a healthy avocado tree allocated in an area affected by avocado white root rot (1), a fungal disease caused by the soilborne phytopathogen *Rosellinia necatrix* (2). The bacterial isolation was carried out from avocado root samples, with further isolation of different nutrient media with cycloheximide (100 μg/ml) to avoid fungal growth interference. *Pseudomonas chlororaphis* PCL1601 formed opaque and light-yellow colonies when grown on solid nutrient medium, and the AQ: A colonies were fluorescent when grown in King's B (KB) medium. Furthermore, PCL1601 presented antagonistic activity against several soilborne pathogens, such as *Fusarium oxysporum* and *Rhizoctonia solani*, but especially to the avocado soilborne pathogens *R. necatrix* and *Phytophthora cinnamomi* (1). Additionally, *P. chlororaphis* PCL1601 showed biological control activity against *R. necatrix* on avocado and to *F. oxysporum* f. sp. *radicis-lycopersici* on tomato (1). This strain is able to produce some antimicrobial compounds, such as hydrogen cyanide (HCN), phenazine-1-carboxylic acid (PCA), and phenazine-1-carboxamide (PCN) (1).

Here, we report the draft genome sequence of *P. chlororaphis* PCL1601. Genomic DNA of *P. chlororaphis* PCL1601 was extracted with the PowerSoil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) after overnight growth in liquid King's B medium at 25°C. Genome sequencing was performed at ChunLab, Inc. (Seoul, South Korea) using the Pacific Biosciences 20 K method. Sequencing depth was 223.26× coverage of the genome, which was assembled *de novo* into 25 contigs with the PacBio SMRT Analysis pipeline version 2.3.0 (ChunLab, Inc.). The resulting draft genome sequence was ordered using the genome sequence of *Pseudomonas chororaphis* PA23 as the template (3). The resulting draft genome sequence was annotated with the NCBI Prokaryotic Genome Annotation Pipeline. Additionally, the secondary metabolite- and antibiotic-encoding gene clusters were predicted with antiSMASH (4).

The draft genome of PCL1601 is 6,755,444 bp in length, containing a G+C content of 64% and 5,897 predicted coding sequences, 17 rRNAs, and 68 tRNAs, features similar to those previously described for the biocontrol strain *P. chlororaphis* PCL1606, also isolated from avocado rhizosphere (5). However, genome annotation displayed a AQ: B higher range of putative genes involved in general metabolism (carbohydrates, amino

acids, lipids, etc.) and transport (such as inorganic ion transport and metabolism, intracellular trafficking, secretion, and vesicular transport). Using antiSMASH, we found 13 potential biosynthetic gene clusters potentially involved in secondary metabolite production, highlighting the phenazine biosynthetic gene cluster, but also bacteriocins ($n = 4$), siderophores ($n = 2$), and nonribosomal peptide synthetases (NRPS; $n = 2$), most of them displaying architecture (higher than 90%) similar to other biosynthetic operons also described in other *P. chlororaphis* strains. The remaining clusters have lower homologies and need further characterization.

Accession number(s). This whole-genome shotgun project has been deposited in GenBank under the accession no. MSCT00000000 (from MSCT01000001 to MSCT01000025). The version described in this paper is the first version, MSCT01000000.

ACKNOWLEDGMENTS

This work was supported by Plan Nacional I–+–D+I from Ministerio de Economía (MINECO) (grants AGL11-30354-C02-01 and AGL14-52518-C2-IR), cofinanced by FEDER funds (EU). C. Vida was supported by a Ph.D. fellowship from the FPI program of MINECO.

REFERENCES

1. Cazorla FM, Duckett SB, Bergström ET, Noreen S, Odijk R, Lugtenberg BJJ, Thomas-Oates JE, Bloemberg GV. 2006. Biocontrol of avocado dematophora root rot by antagonistic *Pseudomonas fluorescens* PCL1606 correlates with the production of 2-hexyl 5-propyl resorcinol. Mol Plant Microbe Interact 19:418–428. https://doi.org/10.1094/MPMI-19-0418.
2. Pliego C, López-Herrera C, Ramos C, Cazorla FM. 2012. Developing tools to unravel the biological secrets of *Rosellinia necatrix*, and emergent threat to woody crops. Mol Plant Pathol 13:226–239. https://doi.org/10.1111/J.1364-3703.2011.00753.X.
3. Loewen PC, Villenueva J, Fernando WG, de Kievit T. 2014. Genome sequence of *Pseudomonas chlororaphis* strain PA23. Genome Announc 2(4):e00689-14. https://doi.org/10.1128/genomeA.00689-14.
4. Weber T, Blin K, Duddela S, Krug D, Kim HU, Bruccoleri R, Lee SY, Fischbach MA, Müller R, Wohlleben W, Breitling R, Takano E, Medema MH. 2015. antiSMASH 3.0–a comprehensive resource for the genome mining of biosynthetic gene clusters. Nucleic Acids Res 43:W237–W243. https://doi.org/10.1093/nar/gkv437.
5. Calderón CE, Ramos C, de Vicente A, Cazorla FM. 2015. Comparative genomic analysis of *Pseudomonas chlororaphis* PCL1606 reveals new insight into antifungal compounds involved in biocontrol. Mol Plant Microbe Interact 28:249–260. https://doi.org/10.1094/MPMI-10-14-0326-FI.

Taking together our results, we showed the positive effect of application of composted almond shells as organic amendment in biological control of avocado soil-borne pathogen *Rosellinia necatrix*. In this work, we assigned this biocontrol activity to soil microbial community, where different groups of *Gammaproteobacterias*, including *Pseudomonas* spp., were naturally selected. Isolation of culturable members from the suppressive soil of *Pseudomonas* spp., *Serratia* spp. and *Stenotrophomonas* spp., showed the ability of these microorganisms to control the disease index cause by the pathogen, both in avocado roots as in wheat root, using different biological control methods. Due to the importance of genus *Pseudomonas* sp. in this suppressive soil, and using *Pseudomonas* spp. previously described by their biocontrol activity against *R. necatrix*, we design a bacterial consortium in order to improve the knowledge of the putative community interactions that occur during biological control process.